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(f) permitting growth of transgenic plant cells into whole plants or suspensions; and

(g) extracting a quantity of human coagulation factor VIII from the plant cells.

REMARKS

Receipt is acknowledged of the captioned action, in which the examiner rejected claims 1-10, 13-15 and 18-23 allegedly for non-enablement and lack of written description, and claim 23 for indefiniteness. The examiner also objected to claims 3, 5 and 23 for failing to capitalize and italicize "*Agrobacterium*", and claim 12 for being dependent on a rejected base claim.

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and for the reasons which follow.

Status of the claims

In this response, applicants amended claims 3, 5, 6, 12 and 23. Claims 3, 5, 6 and 23 were amended to correct a typographical error and claim 12 was rewritten in independent form. In the action, the examiner indicated that the amendment to claim 12 will render the claim allowable (office action at 2). Upon entry of this amendment, claims 1-10, 12-15 and 18-23 will be under examination.

35 U.S.C. § 112, ¶1

The examiner rejected claims 1-10, 13-15 and 18-23 on the grounds that the claims are allegedly not enabled and fail to meet the written description requirement. In particular, the examiner asserted that the specification "does not reasonably provide enablement for methods of expression of any encoding sequence having any fragment and/or modification of human or chimera coagulation factor VIII" (office action at 6). The examiner relied on the erroneous belief that "one of skill in the art would not have known what fragments or modified versions of the disclosed human coagulation factor VIII would have been suitable for expression in plants of a bioactive protein...at the time the invention was made" (office action at 5). Applicants respectfully disagree.

Under §112, the application must explain how to "make and use" the claimed invention. The courts have interpreted this statute to mean that the specification must teach the skilled artisan how to practice the invention without undue experimentation. *See In re Wands*, 858 F.2d 731 (Fed. Cir. 1988); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986). Thus, the test is not whether experimentation is necessary, but whether any experimentation would be undue in

view of what type and amount of experimentation is typical in the area. *See In re Wands*, 858 F.2d at 736-37 ("Enablement is not precluded by the necessity for some experimentation such as routine screening."). *See also* MPEP §2164.01.

Furthermore, the law does not require a patent applicant to exemplify every species in a genus, in order to show enablement of that genus. *See Amgen, Inc. v. Chugai Pharm. Co., Ltd.*, 927 F.2d 1200, 1213 (Fed. Cir. 1991). Rather, the specification must provide sufficient guidance to allow practice of the invention without undue experimentation. Fulfillment of the requirements of §112 does not require that the skilled artisan be able to predict, with certainty, which embodiments of the claimed invention would be enabled. The specification need only describe procedures that can be practiced, without undue experimentation, to determine which embodiments are encompassed by the claims. *See e.g. Wands*, 858 F.2d 731.

The question of what degree of experimentation is permissible is generally based upon the inquiry of whether the amount of experimentation would be burdensome when viewed in light of the particular area of technology involved and the level of skill in that field of technology. These factors include

> (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.
>
> *Wands*, 858 F.2d at 737.

In the instant invention, one of skill in the art would have known at the time the application was filed which fragments and/or modifications would be suitable for use in the present invention. Indeed, scientific literature was available at the time of filing that describes the production of heterologous proteins in animal cell hosts, thereby teaching details of cloning and expression of both factor VIIIa (inactivation resistant coagulation factor VIII) and B-domain deleted factor VIII (now marketed as Refacto®). *See*, Exhibit A. Accordingly, the quantity of experimentation required to identify which nucleotide sequences encodes a functional human factor VIII protein, or which functional fragments would be suitable for use in the present invention, was not undue, but well within the level of the skilled artisan at the time the invention was made

For example, Eaton describe a factor VIII deletion mutant wherein residues 797 through 1562 were omitted by standard molecular biology techniques (Eaton *et al.*, *Biochem.* 25:8343 (1986)), and

Toole teach factor VIII molecules missing 581 or 880 amino acids in the B domain (Toole *et al.*, *P.N.A.S. USA* 83:5939 (1986)). Similarly, large B-domain deletions, such as amino acid residues 760 to 1639, also yielded a protein with similar specific activity compared to the wild-type molecule (Pittman *et al.*, *Blood*, 81:2925 (1993)). It is important to recognize that these modified factor VIII proteins retained procoagulant factor VIII activity.

Furthermore, Pipe describe cloning and expression of factor VIIIa (Pipe *et al. P.N.A.S. USA* 94:11851 (1997)). In particular, Pipe teaches use of missense mutations at thrombin (Arg-740) and activated protein C inactivation cleavage sites to provide greater resistance to proteolysis. This reference also describes the deletion of residues 794-1689 in factor VIII, so that the A2 domain is covalently attached to the light chain.

The examiner further stated that "[t]he fact that the full-length human coagulation factor protein was expressed as per applicants *[sic]* showing[,] does not mean that any fragment or modification thereof will be able to be expressed to make a 'bioactive' protein in plants" (office action at 7). To support this assertion, the examiner relies on Cramer *et al.*, saying that expression of human proteins in plants "is still not a predictable event" (office action at 8-9) and Lollar *et al.*, providing that "factor VIII is activated by thrombin to form a heterotrimer" (office action at 9). None of these references, however, affirm that biologically active fragments and deletions of factor VIII cannot be expressed in plants, especially given that expression of the full-length gene in a plant cell has been shown.

The examiner overlooks the extensive guidance and teachings provided in applicant's own specification for expression of bioactive Factor VIII in transgenic cells. Furthermore, the examiner fails to take into consideration the actual state of the art at the time the invention was made.

Additionally, the examiner asserted that the claims read on any length and any modification of human coagulation factor VIII and that these modifications are "not adequately described in the specification as filed" (office action at 4). However, in levying a written description rejection, an examiner has the burden of presenting by a preponderance of the evidence why a person skilled in the art would not recognize in an applicant's disclosure, a description of the invention defined by the claims. *See In re Wertheim*, 541 F.2d 257, 263 (CCPA 1976). Applicants assert that the examiner has failed to meet her burden.

The fundamental factual inquiry regarding the adequacy of disclosure is whether the application conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the claimed invention. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991). To provide descriptive support, it is not necessary that the application describe the claim limitations exactly. *See e.g. In re Lukach*, 442 F.2d 967, 969 (CCPA 1971)([T]he invention claimed does not have to be described *in ipsis verbis* in order to satisfy the description requirement of § 112.) Rather, the application need only be sufficiently clear that persons of skill in the art would recognize that the applicant had possession of the claimed invention. *See In re Wertheim*, 541 F.2d at 263. Thus, the written description requirement is satisfied when each claim limitation is supported explicitly, implicitly or inherently in the originally filed disclosure. *See* Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112 ¶1, "Written Description" Requirement, 66 Fed. Reg. 1099 (2001).

In the instant case, it is clear that applicants were in possession of the claimed invention at the time of filing. The specification contemplates fragments and/or modifications to human coagulation factor VIII, as factor VIII is defined as "refer[ring] to human like proteins possessing human factor VIII sequence identity *or* human factor VIII-like procoagulation or coagulant activity" (specification at 1, lines 18-21, emphasis added). Therefore, applicants provided a detailed description of the claimed methods and therefore complied with the written description requirement.

Therefore, in view of the teachings in the specification and the references cited herein, it is apparent that one of skill in the art would know what modifications to human coagulation factor VIII can be made for use in the present invention.

35 U.S.C. § 112, ¶2

The examiner rejected claim 23 for alleged indefiniteness, asserting that the phrase "modifying a coagulation factor VIII a sequence encoding human coagulation factor VIII for subcloning into a plant expression vector" is indefinite (office action at 3). Accordingly, applicants amended claim 23 to delete the phrase "coagulation factor VIIIa".

CONCLUSION

As the above-presented remarks address and overcome the rejections presented by the examiner, withdrawal of the rejections and allowance of the claims are respectfully requested. Should Examiner Schmidt believe that further discussion of any remaining issues would advance the

prosecution, a telephone call to the undersigned, at the telephone number listed below, is courteously invited.

Respectfully submitted,

Date February 10, 2003

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

3. (Amended) The method as recited in claim 1, wherein transferring is by **[agrobacterium]** *Agrobacterium* mediated transformation.

5. (Amended) The method as recited in claim 3, wherein **[agrobacterium]** *Agrobacterium* mediated transformation comprises the steps of:

(a) introducing said **[placing the subcloned]** plant expression vector into **[to an agrobacterium]** *Agrobacterium*;

(b) co-cultivating the **[agrobacterium]** *Agrobacterium* containing the subcloned plant expression vector with the plurality of plant cells.

6. (Twice Amended) A method of producing an active human coagulation factor VIII from plant cells, comprising the steps of:

(a) introducing a sequence encoding human coagulation factor **VIII [for production of human coagulation factor VIII]** into a plant expression vector **[in the plant cells]**;

(b) transforming **[obtaining a positive transformant of the]** plant cells with said plant expression vector **[, the positive transformant carrying genetic material encoding the human coagulation factor VIII]**;

(c) cultivating said transformed cells**[the positive transformant]**; and

(d) obtaining the human coagulation factor VIII.

[(b) transferring the subcloned plant expression vector into a plurality of plant cells;

(c) selecting a plurality of positive transformants from the plurality of plant cells on an antibiotic selective media;

(d) growing the plurality of plant cells in whole plants or suspensions; and

(e) extracting and purifying the human coagulation factor VIII from the plurality of plant cells.]

12. (Amended) **[The method as recited in claim 6,]** A method of producing an active human coagulation factor VIII from plant cells, comprising the steps of:

(a) introducing a sequence encoding human coagulation factor VIII into a plant expression vector;

(b) transforming plant with said plant expression vector;

(c) cultivating said transformed cells; and

(d) obtaining the human coagulation factor VIII,

wherein said **[encoding]** sequence encodes a full length of said human coagulation factor VIII.

23. (Twice Amended) A method of producing an active human coagulation factor VIII using **[an agrobacterium]** *Agrobacterium*-mediated transformation, comprising:

(a) modifying a **[coagulation factor VIII a]** sequence encoding human coagulation factor VIII for subcloning into a plant expression vector;

(b) subcloning said **[the]** encoding sequence into said **[the]** plant expression vector;

(c) transferring the plant expression vector to **[agrobacterium]** *Agrobacterium*;

(d) co-cultivating **[a portion of the transgenic]** plant cells with said **[the agrobacterium]** *Agrobacterium*;

(e) selecting positive transformants from the co-cultivated culture on a selection medium **[an antibiotic selective media]**;

(f) permitting growth of transgenic plant cells into **[in]** whole plants or suspensions; and

(g) extracting a quantity of human coagulation factor VIII from the plant cells.

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Haemophilia (1998), 4, 370–379

Can we improve on nature? "Super molecules" of factor VIII

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Summary. Treatment of haemophilia A requires frequent infusion of plasma- or recombinant-derived factor VIII. This regimen is limited due to the high cost and inconvenient access to peripheral veins. In addition, patients frequently develop inhibitory antibodies that limit available therapeutic regimens. Two major advances in factor VIII research over the past 15 years were the ability to isolate homogeneous preparations of factor VIII and the isolation of the factor VIII gene that provided for a detailed biochemical and structural characterization of the factor VIII molecule. With an increased understanding of the requirements for factor VIII function, studies have attempted to produce improved factor VIII molecules for replacement therapy. These findings have produced forms of factor VIII that are more efficiently produced, that are less immunogenic, and that have higher specific activity. The future will see the engineering of novel factor VIII molecules with increased therapeutic efficiency while minimizing inhibitor antibody development. In addition, there are now structural models of factor VIII available that should in the future direct development of novel peptidomimetics that may eventually overcome the requirement for replacement therapy with factor VIII protein.

Keywords: Factor VIII, thrombin, activated protein C, inhibitor antibodies.

Haemophilia A is an X-chromosome-linked bleeding disorder affecting 1/5,000 males that results from a deficiency or abnormality in the plasma protein, factor VIII. Although the crucial role of factor VIII in haemostasis was realized in 1937 [1], a detailed biochemical and structural characterization of factor VIII was only initiated within the last 15 years. In the past, treatment of haemophilia A involved frequent infusion of preparations of factor VIII concentrates derived from human plasma. Although this replacement therapy is effective in controlling bleeding episodes, significant problems exist. First, patients are at risk of blood-borne virally transmissible diseases. The risk of virus infection was significantly reduced by monoclonal antibody purification of factor VIII from human plasma and the development of recombinant-derived factor VIII. However, these improvements have greatly increased the cost of treatment. Second, due to the high cost of factor VIII and the limited access to peripheral veins, patients are generally treated episodically on a demand basis as opposed to prophylactically. A consequence of this therapeutic regimen is chronic bleeding into the joints leading to tissue damage later in life. Finally, about 15% of patients develop inhibitory antibodies to factor VIII. It is likely that solutions to the present limitations in haemophilia therapy will result from further advances in our knowledge about factor VIII. Recombinant DNA technology now provides the ability to design specific changes into the factor VIII gene to derive novel and improved forms of factor VIII. The ability to engineer factor VIII with specific alterations has led to a greater understanding of the regulation of factor VIII expression and its activity and now provides avenues to engineer factor VIII to produce improved proteins for therapeutic use.

Factor VIII structure and function

Factor VIII functions in the intrinsic pathway of blood coagulation as a cofactor to accelerate the activation of factor X by factor IXa that occurs on a phospholipid surface in the presence of calcium ions. The factor VIII amino acid sequence deduced from the cloned cDNA identified that the molecule is synthesized as a single-chain polypeptide having the domain structure A1-A2-B-A3-C1-C2 [2, 3] and upon secretion from the cell is processed to a heterodimer consisting of a carboxy-terminal derived light chain of 80 kDa in a metal-ion

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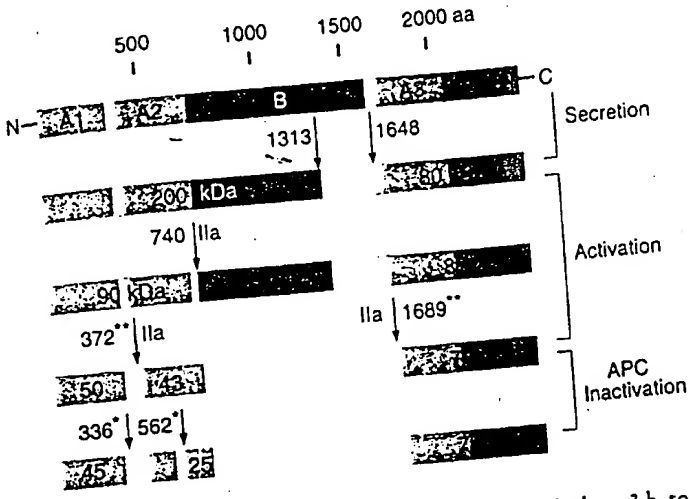


Fig. 1. Domain structure and processing of factor VIII. The structural domains of factor VIII are depicted: A1 domain (1-336), A2 domain (372-740), B domain (740-1648), A3 domain (1690-2020) and the C domains (2020-2332). Three regions rich in acidic amino acid residues between domains A1 and A2, A2 and B, B and A3 are indicated by hashed shading. Intracellularly, factor VIII is cleaved within the B-domain to generate a 200 kDa peptide and the 80 kDa light chain. The two cleavages required for thrombin activation are indicated by **. Sites of activated protein C cleavage are indicated by *.

dependent association with a 200 kDa amino-terminal derived heavy chain fragment (Fig. 1). The domain structure of factor VIII is identical to that of the homologous coagulation factor, factor V [4, 5]. The A domains within factor VIII have 40% amino acid identity with each other and to the A domains of factor V, as well as with the copper binding protein ceruloplasmin [6], suggesting the A domains may be involved in metal ion binding. The C domains exhibit 40% identity to the C domains of factor V, and with proteins that bind glycoconjugates and negatively charged phospholipids [7]. The B-domain is encoded by a single exon and exhibits little homology to the factor V B domain [8, 9]. In plasma, the factor VIII light chain is bound by non-covalent interactions to a primary binding site in the amino terminus of von Willebrand factor (vWF).

The observation that haemophilia A offers protection from ischaemic heart disease [10] and suggestions that elevated factor VIII may be associated with thrombotic disease [11] provides an incentive to understand the mechanism by which factor VIII levels are regulated in plasma. In plasma, vWF circulates as a heterogeneous multimer comprised of 2 to 100 subunits. Although each monomeric vWF molecule contains one factor VIII binding site, *in vitro* binding studies yielded conflicting data for factor VIII:vWF monomer ratios of 1:1 [12], 1:4 [13], 1:10 [14] to as low as 1:70 high affinity binding sites [15, 16]. As different reagents, protein concentrations, and assays were used for these studies, the source for the difference remains unknown. However, the ratio of circulating factor VIII to vWF observed *in vivo* is tightly maintained at 1:50 [17]. Any change in plasma vWF level is coupled with a concordant change in the factor VIII level. The infusion of vWF into vWF deficient patients immediately elevates factor VIII levels to above those observed in normal individuals [17–20]. The presence of vWF increases the plasma half-life of factor VIII from 2–3 h to 12–14 h [21, 22]. Since factor VIII clearance from the circulation is dependent on the FVIII-vWF interaction, it is unlikely that it will be possible to engineer a factor VIII molecule that retains both vWF binding affinity and increased plasma half-life.

In vitro studies demonstrated that vWF regulates factor VIII activity through additional mechanisms: (1) vWF prevents activation of factor VIII by factor Xa [23], whereas it has no effect on activation of factor VIII by thrombin [24, 25]; (2) vWF prevents inactivation of factor VIII by activated protein C [26, 27]; (3) vWF prevents binding of factor VIII to phospholipids [28, 29] and to thrombin-activated platelets [14]; and (4) vWF is required to promote stable accumulation of factor VIII upon secretion into the medium when factor VIII is expressed in mammalian cells in culture [30–32]. Although primary interactive binding sites within the factor VIII light chain (residues 1680–1689 [33, 34] and the C2 domain [35, 36]) and vWF (residues 1–272 [37–39]) have been identified, there are likely multiple contacts that are required to mediate the multitude of effects that vWF has on factor VIII.

In vivo, factor VIII activity is regulated by proteolytic activation as well as inactivation. Upon thrombin activation of factor VIII there is a rapid 30-fold increase and subsequent first-order decay of procoagulant activity. The activation coincides with proteolysis of both the heavy and light chains of factor VIII and release from vWF (Fig. 1) [40–42]. Cleavage within the heavy chain after arginine residue 740 generates a 90 kDa polypeptide that is subsequently cleaved after arginine 372 to yield 50 and 43 kDa polypeptides. Concomitantly, the 80 kDa light chain is cleaved after arginine residue 1689 to generate a 73 kDa polypeptide. Thus, thrombin-activated factor VIII is composed of a heterotrimer of the 50, 43, and 73 kDa fragments [43–45].

Factor VIII and factor VIIIa are both inactivated by

activated protein C cleavage after residues 336 [40, 46] and/or 562 [47], and this mechanism of inactivation appears physiologically significant since protein C deficiency is associated with thrombotic events [48, 49]. However, the first order decay of procoagulant activity for thrombin-activated factor VIIIa observed *in vitro* does not correlate with any specific proteolytic event [50, 51]. A detailed characterization of thrombin-activated factor VIIIa was hampered due to its marked instability. Protein concentration and pH are important factors for isolation of stable thrombin-activated factor VIIIa [43, 52]. However, presently it is not possible to isolate a stable preparation of human factor VIIIa at physiological pH and concentration that would be suitable for functional analysis in biochemical and biological assay systems. Most data support the conclusion that loss of procoagulant activity after thrombin activation results from a reversible dissociation of the 43 kDa A2-domain polypeptide from the heterotrimer which occurs at physiological pH [44, 45, 52, 53]. The specific activity of porcine factor VIIIa, depending on its concentration, is 2–10-fold higher than human factor VIIIa and this correlates with a lower dissociation rate constant of the A2-domain polypeptide with the thrombin-activated heterotrimer [52, 53].

Potential for improved factor VIII molecules through genetic engineering

Recombinant DNA technology provides unique approaches that may yield improved therapeutic regimens for haemophilia A. These approaches include: (1) alterations that improve factor VIII expression, thereby reducing potential cost of treatment and making prophylactic treatment feasible; (2) alterations that reduce factor VIII immunogenicity, thereby reducing potential for inhibitor antibody development; (3) alterations that improve factor VIII specific activity or increase the half-life of activated FVIII in plasma, thereby reducing the amount of protein required for therapeutic use; and (4) development of oral acting compounds that mimick the action of factor VIII. Each of these approaches will be discussed in turn.

Alterations that improve factor VIII expression

Although most evidence supports that the hepatocyte is the cell type that produces factor VIII *in vivo* [54–58], there are no known established or primary cell lines that express factor VIII. Thus, our knowledge of factor VIII expression is derived from interpretation of results from expression of the cDNA from expression vectors in transfected mammalian cells. Expression of factor VIII in these transfection systems is 2–3 orders of magnitude lower than that observed with other genes using similar vectors and approaches. Studies have identified at least three reasons for the low level of expression [59]: (1) the factor VIII mRNA is inefficiently expressed, (2) the primary translation product is inefficiently transported from the endoplasmic reticulum (ER) to the Golgi apparatus, and (3) high levels of vWF are required in the conditioned medium to promote stable accumulation of factor VIII.

Factor VIII expression upon transfection or infection of cells in culture is limited in the ability to produce factor VIII mRNA and to efficiently secrete factor VIII protein from the cell. One of the most significant observations concerning the ability to improve factor VIII expression was that deletion of the middle 1/3 of the coding region, the B-domain, yielded a molecule that was expressed at significantly greater levels than wild-type factor VIII [60]. The increased expression was attributed to a greater increase in the factor VIII mRNA level and corresponding factor VIII protein synthesis [25]. One of these B-domain deleted forms of factor VIII (termed Refacto) is now presently under study in clinical protocols in humans. Refacto has a 2–3-fold increased specific activity over wild-type recombinant factor VIII. Results of preliminary preclinical and clinical studies were recently presented in August of 1996 [61]. The pharmacokinetic parameters were indistinguishable from wild-type factor VIII upon infusion into animal models as well as human patients. However, there was a slightly greater volume of distribution for the B-domain deleted factor VIII, most likely due to its smaller size. Preliminary clinical studies demonstrated an excellent response upon infusion into 87 previously treated haemophilia A patients with no detectable inhibitor development or adverse reactions. At the time of reporting, 43 previously untreated patients were treated with Refacto with an incidence of inhibitor development not significantly different than studies with recombinant wild-type factor VIII. Although preliminary, these studies suggest that deletion of 1/3 of the factor VIII molecule does not significantly change its *in vivo* pharmokinetic, immunological, or functional properties and demonstrates the feasibilty to produce improved factor VIII molecules. The potential benefits from Refacto include a formulation that will be human serum albumin free, a smaller injection volume, infusions containing less protein, and finally, because of its smaller size, the potential to develop a continuous delivery formulation that can be used prophylactically.

Although proteins can fold into correct tertiary conformations *in vitro* [62], additional factors such as protein chaperones are required to assist protein folding *in vivo*. Our work over the past 10 years demonstrated that the inefficient secretion of factor VIII correlated with interaction with the protein chaperone identified as the immunoglobulin binding protein (BiP) which is the same

highlighted text

as the glucose-regulated protein of 78 kDa (GRP78) [63] within the lumen of the ER [64, 65]. BiP is a member of the heat-shock protein family which exhibits a peptide-dependent ATPase activity [66] and for which expression is induced by the presence of aberrantly folded protein or unassembled protein subunits within the ER [67, 68]. Factor VIII release from BiP and transport out of the ER required high levels of intracellular ATP [69]. In contrast, the homologous coagulation protein, factor V, did not detectably associate with BiP and did not require high levels of ATP for secretion [70]. Through expression of chimeric cDNAs, it was possible to localize the sequences within factor VIII that inhibit secretion. Exchange of a 110 amino acid region within the A1-domain improved secretion of the molecule and this chimeric protein displayed a reduced interaction with BiP [71]. However, the secreted protein was not active, and this correlated with dissociation between the heavy and light chains. Mutation of single residues within this region identified that a single amino acid change at Phe309 to Ser (the homologous residue present in factor V) improved factor VIII secretion by 3-fold; however, the secreted protein had a specific activity indistinguishable from wild-type factor VIII [72]. In addition, the Phe309Ser mutant factor VIII displayed a reduced requirement for ATP for secretion, suggesting a reduced interaction with BiP. These results demonstrate that mutation of a single residue in factor VIII can influence chaperone interaction to improve the secretion of factor VIII. The findings provide needed information on what sequences are responsible for BiP binding, and will also have practical importance for improving factor VIII expression. The increased expression will facilitate the eventual goal of somatic cell gene therapy for haemophilia A.

Alterations that reduce immunogenicity of factor VIII

One of the major limitations with present replacement thereapy is the development of inhibitor antibodies to factor VIII. Once inhibitor antibodies develop, several strategies are available to provide effective haemostasis. These include the use of porcine factor VIII, bypass therapy with recombinant factor VIIa, and high dose factor VIII with or without immunosuppressive agents in order to induce a state of nonresponsive tolerance. However, it should also be possible to engineer factor VIII to make it less immunogenic. One particular strategy could involve the modification of factor VIII to prevent exposure of antigenic epitopes. For example, covalent modification by polyethylene glycol attachment to lysine residues can both reduce immunogenicity and increase plasma half-life [73]. This is most dramatically demonstrated for modification of adenosine deaminase for the treatment of severe combined immunodeficiency disease [74]. However, to date there are no reported results on the successful modification of factor VIII by polyethylene glycol attachment.

An alternate strategy would be to produce a less immunogenic factor VIII through genetic engineering. This first requires identification of the regions within factor VIII that elicit an immune response and then selective modification of those regions by mutagenesis. Studies have established that the most common factor VIII epitopes that induce inhibitory antibodies are localized to the A2 domain (residues 373–740) and the C2 domain (residues 2173–2332) of factor VIII [75–77]. An additional epitope has also been identified that may be localized to the A3 domain [78, 79]. Since porcine factor VIII can be used to treat inhibitor patients [80, 81], it is likely that the epitopes recognized by anti-human factor VIII inhibitory antibodies are not present in porcine factor VIII. This observation provided the impetus for Lollar and coworkers to prepare human and porcine factor VIII chimeric molecules in order to elucidate what amino acids are responsible for the antigenic differences between human and porcine factor VIII. The results identified a limited number of residues between 484 and 508 within the A2 domain of human factor VIII that contribute significantly to the antibody response [82].

Once critical residues are identified that are responsible for the immunogenicity of human factor VIII, it is possible to alter those residues in the hope of reducing the immunogenicity. In particular, residues that present as strong epitopes for immune response are frequently surface exposed and either positively or negatively charged. By mutation of those amino acids that have charged side chains to alanine, an amino acid that lacks side chains, it may be possible to reduce the immunogenicity of factor VIII. The feasibility of this concept was recently tested by mutation of those residues in the human factor VIII A2 domain that were implicated to elicit inhibitory antibodies to alanine (Fig. 2) [83]. Resultant molecules retained procoagulant activity, but did demonstrate significantly reduced inhibition to an inhibitory monoclonal antibody that reacts with wild-type human factor VIII. This alanine scanning mutagenesis approach identified Tyr487 as a residue that is critical for the recognition by anti-human factor VIII A2-inhibitor antibodies [83]. Once all the immunogenic regions are identified, it may be possible to mutate all the relevant amino acids and yield a molecule with markedly reduced immunogenicity that retains full functional activity.

Alterations that increase the specific activity of factor VIII

The instability of thrombin-activated factor VIII

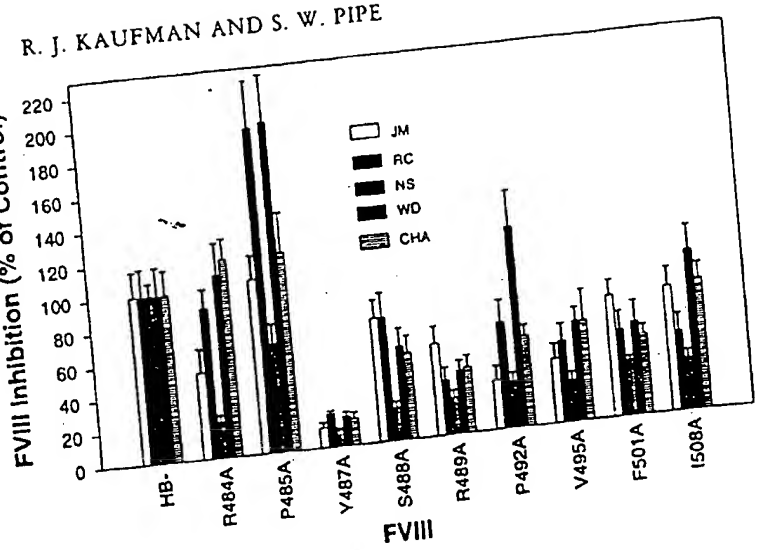


Fig. 2. Alanine-scanning mutagenesis of factor VIII inhibitor epitope. Mutagenesis and expression of B-domain deleted factor VIII was performed and analyzed for inhibition by five different patient inhibitor plasmas as measured by Bethesda assay. For details see [83].

correlates with dissociation of the A2-domain subunit. Therefore, it was considered feasible to derive a more active form of factor VIII if it were possible to minimize dissociation of the A2-domain subunit. To test this hypothesis, experiments were designed to create a form of factor VIII in which the A2-domain was covalently attached to the light chain.

The basis for this approach relied on two observations. First, characterization of cleavage site mutant factor VIII demonstrated that cleavage at residues 372 and 1689 were the only cleavages required for activation of factor VIII activity [84]. Cleavage after 372 was proposed to alter the conformation of the molecule in a manner necessary for cofactor activity [84], whereas cleavage after 1689 was proposed to be required to liberate factor VIIIa from vWF [85] and permit factor VIIIa to interact with negatively charged phospholipids. The second observation was that deletion of residues 741–1689 yielded a molecule (termed 90/73) that displayed significantly reduced binding to vWF and displayed procoagulant activity similar to wild-type factor VIII after treatment with thrombin. Following cleavage by thrombin, the 90/73 factor VIII yielded the 50 kDa/43 kDa/73 kDa heterotrimer that was identical to wild-type factor VIII [86] (Fig. 3A). These observations suggested that if the cleavage between the 740–1690 junction in the 90/73 molecule was prevented, then it should be possible to yield a dimeric factor VIIIa through cleavage by thrombin after residue 372 in which the A2-domain would be covalently attached to the light chain. This molecule may not require cleavage at the amino terminus of the light chain before residue 1690 for activation because it would display significantly reduced binding to vWF. However, when the Arg740 at the junction of the 90kDa and 73 kDa chains was mutated to Lys, the site was not cleaved by thrombin, however the resultant molecule was not active. It was then proposed that the A2-domain may require a spacer so as to attain a conformation that was suitable to develop procoagulant activity. Subsequently, a 54 amino acid spacer from residues 741-794 was inserted and it was observed to yield a molecule that retained factor VIII activity [86]. In order to further increase resistance to inactivation, the resultant molecule was made resistant to cleavage by activated protein C by introducing both Arg336Ile and Arg562Lys mutations. These two mutations were previously shown to inhibit activated protein C inactivation of factor VIII, without affecting its procoagulant activity [87]. The resultant molecule, termed inactivation resistant factor VIII (IR8), had a 5-fold greater specific activity than wild-type factor VIII when measured in a one-stage clotting assay using factor VIII deficient plasma (Fig. 3B). In addition, IR8 displayed 38% of peak activity at 4 hr after activation by thrombin under conditions in which wild-type factor VIII that was completely inactivated after 5 min (Fig. 3C). The results demonstrate the feasibility to produce a form of factor VIII that has elevated specific activity in an *in vitro* clotting assay and that is resistant to inactivation that occurs after thrombin activation and by treatment with activated protein C.

To further characterize the *in vivo* activity of IR8, the factor VIII genetically deficient haemophilic mouse was used [88]. This haemophiliac mouse cannot survive a tail bleed induced by a guillaotine device to remove the last 1 cm of the tail. When as low as 20 ng of wild-type recombinant factor VIII was infused into the mouse tail vein prior to the induced tail bleed, the animal survived (Fig. 4). The reproducibility in survival detected upon recombinant wild-type factor VIII infusion is compromised by difficulty in ensuring the injected factor VIII actually gets into the circulation. However, this model does provide a stringent test for the ability of a given preparation of factor VIII to correct a tail bleed in the

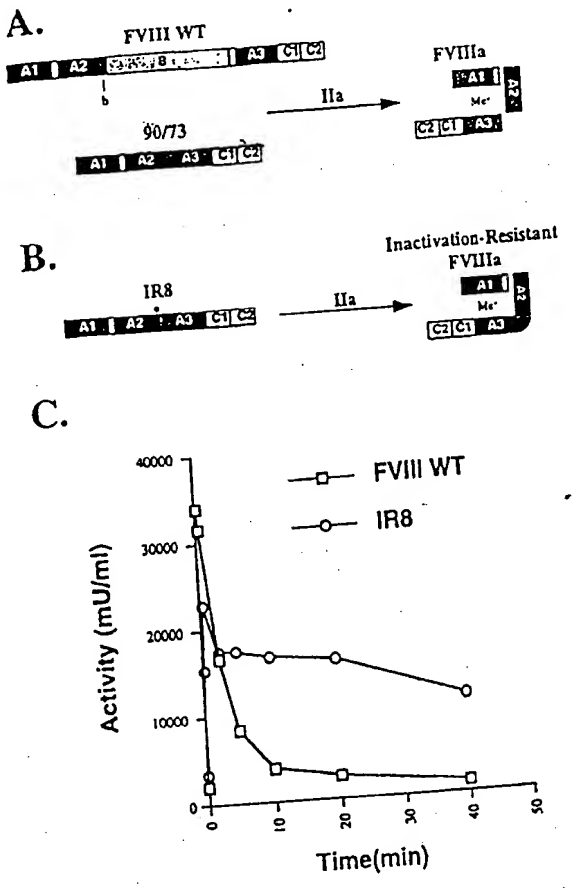
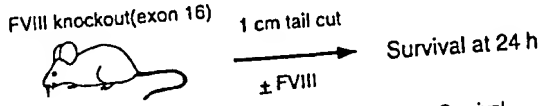


Fig. 3. Inactivation resistant factor VIII. Panel A. Structural domains of factor VIII wild-type (WT) and B-domain deleted factor VIII (90/73) and their predicted factor VIIIa heterotrimeric structure after thrombin (IIa) cleavage. Me+ represents a metal ion necessary for A1 and A3 domain association. Panel B. A representation of IR8 and its predicted heterodimeric subunit structure that results after thrombin activation. *indicates the missense mutation at residue 740 predicting resistance to thrombin cleavage. b indicates 54 amino acids of B-domain retained in the IR8 construct. White boxes represent acidic amino acid rich regions. Panel C. Activation of wild-type and IR8 factor VIII by thrombin. Partially purified proteins (1 nM) were treated with 1 unit/ml thrombin at room temperature and assayed over time for factor VIII activity by the activated partial thromboplastin assay. See [86] for details.



	Injected Antigen(ng)	Survival
control mouse	0	6/6
FVIII knockout + rFVIII	0	0/6
	2	0/3
	20	6/8
	100	5/5
	200	4/5
FVIII Knockout + IR8	10	1/1
	50	4/4

Fig. 4. IR8 displays coagulant activity *in vivo*. Purified wild-type recombinant human factor VIII(rFVIII) or IR8 were infused into the tail vein of anaesthetized genetically deficient mice (factor VIII Exon 16 knockout). One minute following infusion a 1 cm terminal section of the tail was cut. Mice were then observed for evidence of effective haemostasis over a 24 h period. Mice that failed to clot received tail cauterization or, where appropriate, were euthanized. Survivors achieved effective haemostasis in the absence of tail cauterization.

mouse. When IR8 was purified and infused into the haemophilic mouse model, the mouse was able to survive the lethal consequences of the tail bleed. Although we do not know the half-life of IR8 infused into the mouse, it is likely to be significantly shorter than wild-type factor VIII because of its reduced ability to bind vWF. Therefore, these functional data strongly support that the IR8 molecule is at least as effective as wild-type factor VIII in this mouse model system.

The ability to isolate a stable thrombin-activated form of human factor VIIIa will provide a crucial reagent to study the functional significance of VIIIa generation in *in vitro* as well as in *in vivo* studies. The haemostatic efficacy of the more stable thrombin-activated factor VIII heterodimer (IR8) will next be evaluated in a haemophilic dog model [89]. These studies will provide important information concerning the role of the A2-domain dissociation and vWF interaction for factor VIII function *in vivo*. In addition, factor VIII of increased specific activity may have tremendous therapeutic potential by reducing dosage requirements thereby reducing cost of therapy, and reducing the antigenic stimulation to minimize inhibitor antibody development in patients that occurs as a response to factor VIII as a foreign antigen.

Development of oral acting compounds that mimick the action of factor VIII

Our knowledge of the structural requirements for factor VIII activity has dramatically increased since the original isolation of factor VIII protein from human plasma and identification of the factor VIII gene. In addition, a crystal structure of the homologous plasma protein ceruloplasmin is now available [90] that was used to predict the structure of the factor VIII A domains [91]. Additional new insights have come from mutagenesis studies to identify critical regions for factor VIII function and from biochemical analyses to identify interacting regions between factor VIII and factor IXa. Peptide and antibody inhibition data suggest that residues 558–565 and 1778–1840 in factor VIII comprise two sites that interact with the catalytic domain and the first EGF domains of factor IXa, respectively

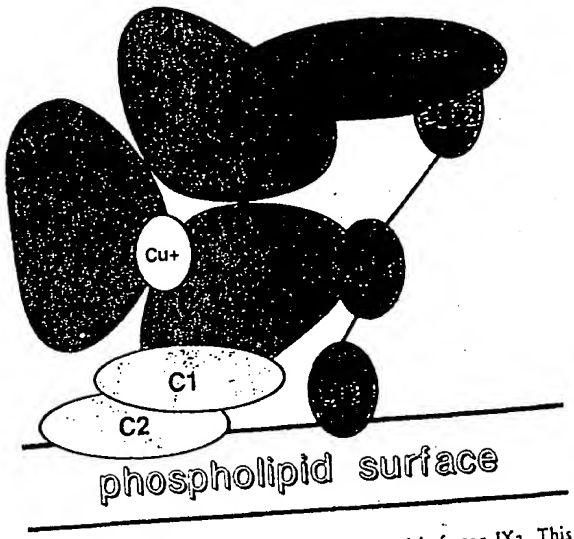


Fig. 5. Model for factor VIIIa interaction with factor IXa. This figure depicts the factor VIII domains A1, A2, A3, C1 and C2 interacting with a phospholipid surface through the C2 domain and the specific interaction of the A2 and A3 domains with factor IXa sites within the serine protease domain and the first epidermal growth factor-like domain. Factor IXa interacts with the phospholipid surface through its region that contains gamma-carboxy glutamic acid residues (gla). Adapted from [91].

[92–95]. With elucidation of the structure of factor IXa [96], it is now feasible to predict how factor VIII may function to enhance the catalytic efficiency of factor IXa. These findings have provided a model where the factor VIII light chain is responsible for complex assembly via the first EGF-like domain in factor IXa, whereas the interaction site with the A2-domain might induce a conformational change within the active site of factor IXa (Fig. 5) [91]. With greater understanding of this conformational change, it may be possible to derive a small peptide, or a peptidomimetic, that mediates the same conformational change. Once a compound is identified, it may be modified to make it more readily delivered by oral administration. These strategies provide enthusiasm for future studies to elucidate the mechanism by which a complex molecule like factor VIII can mediate a specific conformational change in the active site of factor IXa.

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Biochemical, Immunological, and In Vivo Functional Characterization of B-Domain–Deleted Factor VIII

By Debra D. Pittman, Edward M. Alderman, Kathleen N. Tomkinson, Jack H. Wang, Alan R. Giles, and Randal J. Kaufman

Coagulation factor VIII (FVIII) is a cofactor in the intrinsic pathway of blood coagulation for which deficiency results in the bleeding disorder hemophilia A. FVIII contains a domain structure of A1-A2-B-A3-C1-C2 of which the B domain is dispensable for procoagulant activity in vitro. In this report, we compare the properties of B-domain–deleted FVIII (residues 760 through 1639, designated LA-VIII) to wildtype recombinant FVIII. In transfected Chinese hamster ovary (CHO) cells, LA-VIII was expressed at a 10- to 20-fold greater level compared with wildtype FVIII. The specific activity of purified LA-VIII was indistinguishable from wildtype recombinant FVIII and both exhibited similar thrombin activation coefficients. Wildtype recombinant-derived FVIII and LA-VIII also displayed similar timecourses of thrombin activation and heavy chain cleavage. However, compared with wildtype recombinant-derived FVIII, the light chain of LA-VIII was cleaved fivefold more rapidly by thrombin. Addition of purified von Willebrand factor (vWF) did not alter the kinetics of thrombin cleavage or activation of either wildtype recombinant-derived FVIII or LA-VIII. The immunogenicity of LA-VIII was compared with wildtype FVIII in a novel model of neonatal tolerance induction in mice. The results did not detect any immunologic differences between wildtype FVIII and LA-VIII, suggesting that LA-VIII does not contain significant new epitopes that are absent in wildtype FVIII. LA-VIII was tolerated well on infusion into FVIII-deficient dogs and was able to correct the cuticle bleeding time similar to wildtype recombinant factor VIII. In vivo, LA-VIII was bound to canine vWF and exhibited a half-life similar to wildtype recombinant FVIII. These studies support that B-domain–deleted FVIII may be efficacious in treatment of hemophilia A in humans.

FACTOR VIII (FVIII) functions in the intrinsic pathway of blood coagulation as the cofactor for the FIXa-mediated activation of FX.[1] Deficiency of FVIII results in the bleeding disorder, hemophilia A. In plasma, FVIII consists of a heterogeneous sized heavy chain polypeptide extending up to 200 Kd in a metal ion complex with an 80-Kd light chain polypeptide. This complex is stabilized through hydrophobic and hydrophilic interactions with von Willebrand factor (vWF). FVIII is processed from a single-chain polypeptide having the domain structure A1-A2-B-A3-C1-C2.[2,3] The heavy chain is composed of domains A1-A2-B while the light chain is composed of domains A3-C1-C2. The large B domain is encoded by a single large exon[4] and has no detectable homology to any other known genes. The B domain is extensively glycosylated on asparagine, serine, and threonine residues.[5,6] In addition to the primary amino acid sequence, the isolation of the FVIII gene provided the ability to express FVIII in transfected mammalian cells to study its biosynthesis and processing.[5] It was also possible to construct specific mutations within FVIII to study structural requirements for FVIII function.[7-9] Initial studies showed that deletion of the B domain resulted in a functional molecule as tested by in vitro assays and was expressed more efficiently compared with wildtype FVIII.[7,10-12]

Hemophilia A is treated by frequent administration of FVIII preparations derived from pooled human plasma. In the past this therapeutic regimen had associated complications of blood-borne infectious pathogens. Recently, the availability of monoclonal antibody (MoAb) affinity-purified plasma FVIII preparations has significantly reduced risks associated with plasma-borne infectious agents.[13] However, treatment is generally limited on demand basis because of the limited availability and high cost associated with highly purified plasma-derived FVIII. This may be alleviated in the near future by the availability of recombinant-derived human FVIII.[14,15] However, the present manufacturing process for FVIII has limitations because of the inefficiencies of producing, purifying, and characterizing this large heterogeneous glycoprotein. For these reasons it should be advantageous to produce a more efficiently expressed molecule that has reduced heterogeneity to reduce the cost to provide prophylactic therapy for hemophilia A. Here we describe the production and characterization of a B-domain–deleted form of FVIII and show that it has biochemical, immunologic, and in vivo functional properties very similar to wildtype recombinant-derived human FVIII.

MATERIALS AND METHODS

Derivation of LA-VIII–expressing cell lines. Cell clone LA3-5 was obtained by cotransfection with dihydrofolate reductase (DHFR) in the DHFR deficient cell line DUKX B11[16] and selection for growth in the absence of nucleosides and increasing concentrations of methotrexate (MTX) up to 1 μmol/L as previously described.[12] The von Willebrand factor (vWF) expressing cell line PM5F was obtained by coamplification with an adenosine deaminase (ADA) gene and selection for growth in cytotoxic concentrations of adenosine with the ADA inhibitor 2'-deoxycoformycin (dCF) as previously described.[17]

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Aspects of the experiments reported here were presented in abstract form at the 1987 International Congress on Thrombosis and Hemostasis.[39]

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To obtain a cell line that expressed both LA-VIII and vWF, a hybrid was generated by polyethylene glycol induced fusion of LA3-5 (grown in 1 μmol/L MTX) and PM5F (grown in 0.03 μmol/L dCF). The resulting hybrid was subsequently selected for growth in 0.03 μmol/L dCF and 5 μmol/L MTX to yield clone G12. In addition, a clonal cell line expressing higher levels of LA-VIII, designated M18, was derived by coamplification of the LA-VIII coding region contained within the pED expression vector[18] by transfection of the vWF expressing cell line PM5F and selection for resistance to 1 μmol/L MTX and 0.1 μmol/L dCF. The wildtype FVIII and vWF coexpressing cell line C6 was previously described.[19]

For analysis of FVIII and vWF expression, logarithmically growing cells were rinsed with phosphate-buffered saline (PBS) and fed either alpha medium (GIBCO Corp, Grand Island, NY) containing 10% dialyzed fetal calf serum (FCS) or serum-free α medium (medium supplemented with 5 g/L bovine serum albumin [BSA], 5 ng/mL selenium, 5 μg/mL insulin, 5 μg/mL transferrin, 0.1 μg/mL putrescene, 0.01 μmol/L hydrocortisone, 2 mmol/L glutamine, and penicillin-streptomycin [GIBCO]). After 24 and 48 hours the conditioned medium was harvested and centrifuged to remove cellular debris. At that time cell numbers were determined using a Coulter Counter (Hialeah, FL). FVIII activity was assayed in a chromogenic assay that measures the generation of FXa (Kabi Coatest; Kabi Vitrum, Stockholm, Sweden). Quantitation of vWF was performed by an enzyme-linked immunoabsorbant assay (ELISA) using a goat anti-vWF antibody (American Diagnostica, Greenwich, CT) and normal pooled human plasma as a standard (George B. King Biomedical, St Louis, MO).

The synthesis of FVIII was monitored by metabolically labeling Chinese hamster ovary (CHO) cells expressing LA-VIII (M18 and G12) or wildtype FVIII (C6). The cells (growing on a 100-mm^2 dish) were rinsed and fed 1.5 mL of methionine-free minimal essential medium containing 10% dialyzed FCS, 20 μg/mL aprotinin (Sigma Chemical Co, St Louis, MO), 2 mmol/L glutamine, and penicillin-streptomycin. After 15 minutes at 37°C the medium was removed and the cells were fed 1.5 mL of methionine-free medium containing 300 μCi ^{35}S-methionine (1,000 Ci/mmol/L; Amersham, Arlington Heights, IL). After 2 hours, the cells were fed 1.5 mL of complete α medium. Conditioned medium was harvested 4 hours later and processed as described. FVIII was immunoprecipitated with a heavy-chain–specific MoAb F8 coupled to CL-4B Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) and washed as described.[20] Immunoprecipitates were resuspended in 50 mmol/L Tris-HCl pH 7.5, 0.15 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, and 5% glycerol and digested with 10 U/mL thrombin at 37°C for 1 hour. After thrombin digestion, samples were treated with Endoglycosidase-H or N-glycanase according to the manufacturer's specifications (Genzyme, Boston, MA). The polypeptides were separated on a sodium dodecyl sulfate (SDS) 8% low-bis polyacrylamide gel and exposed for autoradiography after treatment with En^{3}hance (DuPont-New England Nuclear, Boston, MA).

Purification of LA-VIII. Logarithmically growing cells were rinsed with PBS and fed serum-free medium. After 48 hours, conditioned medium was collected and FVIII purified by monoclonal affinity chromatography and ion exchange Mono-S and Mono-Q chromatography as previously described.[21] For in vivo efficacy studies, the purification was performed with endotoxin-free reagents and the level of endotoxin in the final purified FVIII was less than 1.5 EU/1,000 units.

The specific activity of FVIII was determined using the one-stage clotting assay[22] or the Kabi Coatest assay. One unit of FVIII is the amount of FVIII in 1 mL of pooled normal human plasma. Protein concentration was determined by BioRad (Richmond, CA). FVIII at 10 μg/mL in 50 mmol/L Tris-HCl pH 7.5, 0.15 mol/L NaCl, 2.5 mmol/L $CaCl_2$, and 5% glycerol was incubated at room temperature with 0.5 μg/mL human thrombin. At short intervals aliquots were removed and clotting activity determined[22] or β-mercaptoethanol (2.5%) and SDS (1%) were added and samples heated at 85°C for 3 minutes. The polypeptides were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver stain (BioRad). Band intensities were determined by scanning with an LKB UltroScan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Where indicated, 30 μg/mL of human vW (Diagnostica Stago, Asnieres-Sur-Seine, France) was added for 2 minutes at room temperature before thrombin treatment.

In vivo studies of FVIII function. Recovery and survival studies and in vivo efficacy were performed in a hemophilic dog model previously described.[23] LA-VIII was compared with wildtype recombinant FVIII by a standardized protocol. Two severely affected homozygous hemophilic animals were used. Both are members of a permanent colony of hemophilic animals maintained at Queen's University.[24] The animals were anesthetized with a rapid acting intravenous barbiturate (Bio-Tal; Boehringer Ingelheim, Burlington, Ontario, Canada) and each animal was allowed to equilibrate for 30 minutes before the infusion of the test material that was delivered by a bolus infusion over 4 minutes into an antecubital vein. Blood sampling was obtained by an in-dwelling 21-gauge butterfly needle inserted into a contralateral antecubital vein that was kept open by a slow intravenous (IV) infusion of isotonic saline for injection. After recovery from anesthesia (2 to 3 hours), subsequent blood samples were obtained by individual venipuncture. In each case a two-syringe technique was used with the first specimen being discarded and subsequent specimens anticoagulated with EDTA for blood counting and buffered citrate (0.1 mol/L sodium citrate, 0.1 mol/L EACA: 9 vol of blood/L vol anticoagulant) for coagulation assays. The test recombinant FVIII preparation was stored frozen at −70°C and thawed at 37°C immediately before the infusion studies were performed. The FVIII coagulant activity of the material infused was assayed using a one-stage assay employing human FVIII-deficient plasma as a substrate and normal canine pooled plasma (10 animals) as a standard. The expected (ie, theoretical) increment in FVIII at 30 minutes postinfusion was calculated according to the formula:

$$\% \text{ Increment in FVIII} = \frac{\text{Dose of FVIII(units)}}{\text{Body Weight(kg)} \times 0.4}$$

Blood samples were obtained immediately before the infusion of the test material and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 16, 24, and 30 hours postinfusion. FVIII functional and antigen assays were performed on each specimen as previously described.[23] Platelet counting was performed using an electronic particle counter as previously described.[24] Fibrinogen assays were performed according to the methods of Clauss.[25] Functional activity in vivo was determined using the cuticle bleeding time (CBT) as previously described.[24] CBTs were performed immediately before the infusion of the test material and then at 30 minutes and 2 hours postinfusion.

To investigate the interaction between the infused recombinant human FVIII and circulating canine vWF, selected plasma specimens were chromatographed on Sepharose 4B (Pharmacia Fine Chemicals) and FVIII antigen assays performed on eluting fractions to determine a shift in molecular weight of material demonstrating FVIII antigen.[23] The column was equilibrated in 0.15 mol/L NaCl, 0.025 mol/L sodium citrate (pH 6.5) and 1% (wt/vol) BSA (Sigma). After equilibration, the test samples were applied and eluted at a flow rate of 4.8 mL/h and 0.4-mL fractions collected and assayed for FVIII antigen and vWF antigen.

The animal studies described had received prior approval by the Queen's University Institutional Animal Care Committee, which operates in accordance with the guiding principles of the Canadian

Council for Animal Care and the International Society of Thrombosis and Haemostasis.[26]

Induction of neonatal tolerance and measurement of antibody. Tolerogenic forms of plasma (pVIII)- or recombinant-derived wildtype (rVIII) or B-domain–deleted (LA-VIII) FVIII were prepared by adjusting the protein concentration to 500 μg/mL in PBS (9 mmol/ sodium phosphate, 0.14 mol/L NaCl, pH 7.4), and centrifuging e resulting solutions at 20,000*g* for 15 minutes at 4°C to minimize rotein aggregates. Immunogenic forms of these FVIII derivatives were prepared by emulsification of the tolerogenic forms in an immunologic adjuvant (RAS-Ribi Adjuvant system: Ribi Immunochemicals, Ogden, UT). Tolerogenic doses (50-μL volumes containing 25 μg FVIII) were administered intraperitoneally to neonatal Balb/CBy mice (Jackson Laboratories, Bar Harbor, ME) within 30 hours of birth to induce a state of antigen-specific immunologic nonresponsiveness. Specificity of the induced nonresponsiveness was determined by monitoring the response to an unrelated protein antigen control (KLH: 5 μg keyhole limpet hemocyanin). Sham-tolerized animals received 50 μL protein-free PBS, and sham-immunized animals received control antigen, but no immunogenic doses of FVIII. All injections subsequent to the administration of the tolerogenic dose were administered subcutaneously, as emulsifications of protein in an immunologic adjuvant according to the schedule in Table 1, section A.

The presence of rFVIII–, LA-FVIII–, and KLH–specific antibodies in serum samples was assessed by a standard ELISA in which 96-well ELISA plates were coated with 100 μL test antigen solution (1 to 3 μg/mL) in carbonate buffer, pH 9.0. Nonspecific protein binding to the well surfaces was minimized by incubation with 300 μL 5% gelatin/PBS at 37°C for 30 minutes. Duplicate serial fivefold dilutions of test sera were added in 300-μL volumes, and the plates were incubated 18 hours at 4°C. Plates were washed four times with 1 mL/well using PBS containing 0.5% Tween 20 (PBST). Specifically bound antibodies were detected using a 300 μL of a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse Ig (heavy and light chain), and developed with o-phenylene diamine solution (5 mg/mL in carbonate buffer pH 9.0). The enzymatic reaction was halted by addition of 4.5 N H_2SO_4, and absorbance at 490 nm was measured. Raw absorbance data was converted to percent maximal signal obtained for the sample, and evaluated by four parameter logistic analysis to provide titration values (dilution at which half-maximal response was observed).

FVIII-specific immunologic response was also monitored in all day 32 samples by Western immunoblot analysis directed against

Table 1. Immunogenicity of LA-VIII in Tolerized Neonatal Mice

	Group A (n = 15) *Sham*-Tolerized, rVIII-Challenged Mice	Group B (n = 9) *Sham*-Tolerized, LA-VIII–Challenged Mice	Group C (n = 9) rVIII-Tolerized, LA-VIII–Challenged Mice	Group D (n = 5) rVIII-Tolerized, rVIII-Challenged Mice	Group E (n = 5) LA-VIII–Tolerized, rVIII-Challenged Mice
Section A					
Day 0: Tolerogen	PBS	PBS	rVIII	rVIII	LA-VIII
Day 7: Control-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS
Day 10: Tolerogen-RAS	5 μg rVIII-RAS	5 μg LA-VIII-RAS	5 μg rVIII-RAS	5 μg rVIII-RAS	5 μg LA-VIII-RAS
Control-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS
Day 16: Test Ag-RAS	5 μg rVIII-RAS	5 μg LA-VIII-RAS	5 μg LA-VIII-RAS	5 μg rVIII-RAS	5 μg rVIII-RAS
Control-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS
Day 24: Test Ag-RAS	5 μg rVIII-RAS	5 μg LA-VIII-RAS	5 μg LA-VIII-RAS	5 μg rVIII-RAS	5 μg rVIII-RAS
Control-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS	5 μg KLH-RAS
Day 32: Exsanguination	+	+	+	+	+
Section B					
Mean d 16 titer *v* KLH (naive ≤ 20)	49 ± 18	45 ± 19	42 ± 12	40 ± 15	44 ± 20
Mean d 16 titer *v* rVIII (naive ≤ 50)	58 ± 17	55 ± 22	52 ± 28†	40 ± 12	52 ± 15
Mean d 16 titer *v* LA-VIII (naive ≤ 50)	48 ± 8	45 ± 18	27 ± 19†	35 ± 14	32 ± 12
Mean d 32 titer *v* KLH	82 ± 18	93 ± 31	78 ± 25† (80 ± 43)	81 ± 26	93 ± 22
Mean d 32 titer *v* rVIII	1,609 ± 485	501 ± 182	65 ± 12† (95 ± 74)	78 ± 38	385 ± 91
Mean d 32 titer *v* LA-VIII	497 ± 522	399 ± 221	46 ± 31† (78 ± 52)	55 ± 24	220 ± 121

Tolerization and challenge were performed as described in Materials and Methods. After 24 days, mice were exsanguinated and sera taken for analysis. Section A: Timetable of tolerance induction for four groups of mice is shown. Section B: The titer of specific Ig reactive with wildtype FVIII (rVIII), LA-VIII, or KLH (the control immunogen in all samples) detected by ELISA as described in Materials and Methods is shown. The values are presented as a mean ± SD (Section B). In group C values are excluding (including) a single mouse with significant detectable antitolerogen response at day 21.

intact or thrombin-cleaved rVIII or LA-VIII. SDS-polyacrylamide gels were loaded with 25 μg FVIII (rVIII), and electrophoresed for 2 hours at 200 mA. Resolved protein components were electrophoretically transferred to nitrocellulose filters (4 hours at 300 mA) using buffer containing 192 mmol/L glycine, 25 mmol/L Tris base, 20% methanol, and 0.05% SDS. The filters were then incubated for 1 hour at 37°C in PBST containing 4% BSA to minimize nonspecific binding of immunologic reagents, then inserted into multichannel immunoblot devices (miniblotter-16; Immunetics, Cambridge, MA). Day 32 samples from each of the test animals were diluted 1:20 in PBST, loaded (200 μL/lane) into the manifold devices, and incubated for 18 hours at room temperature. After this primary incubation step, the devices were disassembled, and the filters washed four times with PBST. The filters were then incubated for 2 hours at room temperature with rabbit anti-murine IgG, IgA, and IgM (heavy and light chain) diluted 1:1,000 in PBST, and washed four times with PBST. Signal was identified using 5 mmol/L $MgCl_2$, 10 μmol/L $ZnCl_2$, 50 mmol/L 2-amino-2 methyl-1,3 propanedial pH 9.7 (Sigma), 0.2 mg/mL nitro blue tetrazolium (Sigma), and 0.2 mg/mL 5-bromo-4 chloro-3 indolyl phosphate (Sigma).

RESULTS

Characterization of LA-VIII produced by CHO cells. Previously, a B-domain deletion molecule of FVIII was constructed that had deleted 880 amino acids from the B domain (residues 760 through 1639).[7] This deletion begins 19 amino acid after the thrombin cleavage site at residue 740 and ends 50 amino acids before the thrombin cleavage site at residue 1689. The new junction of the heavy and light chains results in the juxtaposition of the amino acids asn-ala-thr, a potential N-linked glycosylation site. If this site was used it should prevent exposure of a new epitope at the site of the deletion and thus may reduce potential immunogenicity. The LA-VIII expression vector was introduced into CHO cells with a selectable and amplifiable marker gene, DHFR as described in Materials and Methods. Two cell lines obtained were designated G12 and M18. Both these cell lines also express human vWF at approximately 10 pg/cell/d (Fig 1C), which is required to stabilize FVIII on secretion into the conditioned medium.[5,19] M18 expressed approximately 20-fold greater levels of FVIII than obtained from a similarly engineered wildtype FVIII-expressing cell line C6.[19] Removal of serum reduced the amount of FVIII and vWF accumulation in M18 and G12 cells (Fig 1A). However, for cell line G12, and to a lesser extent M18, this effect was attributable to a slower doubling time in the absence of serum because cellular productivity of LA-VIII (LA-VIII/cell) did not change with removal of serum (Fig 1B).

The structure of polypeptides comprising LA-VIII was studied by labeling cells expressing LA-VIII or wildtype FVIII with ^{35}S-methionine. Conditioned medium was harvested for analysis by immunoprecipitation and SDS-PAGE before and after digestion with thrombin alone or in combination with N-glycanase or endoglycosidase H (Fig 2). FVIII isolated from G12 and M18 cells showed the same spectrum of polypeptides whether analyzed before or after thrombin digestion. The two primary species represented a doublet of the heavy chain (O) and a doublet of the light chain (O). In addition, a small amount of single-chain LA-VIII was detected (*). Pro-vWF and mature vWF that coprecipitated with LA-VIII were also detected migrating at the top of the gel (arrows). In contrast, wildtype FVIII expressed from the cell line C6[19] was composed primarily of the heavy chain migrating at 200 Kd and an 80-Kd light chain. Thrombin digestion of LA-VIII yielded the appropriate thrombin cleavage fragments comigrating

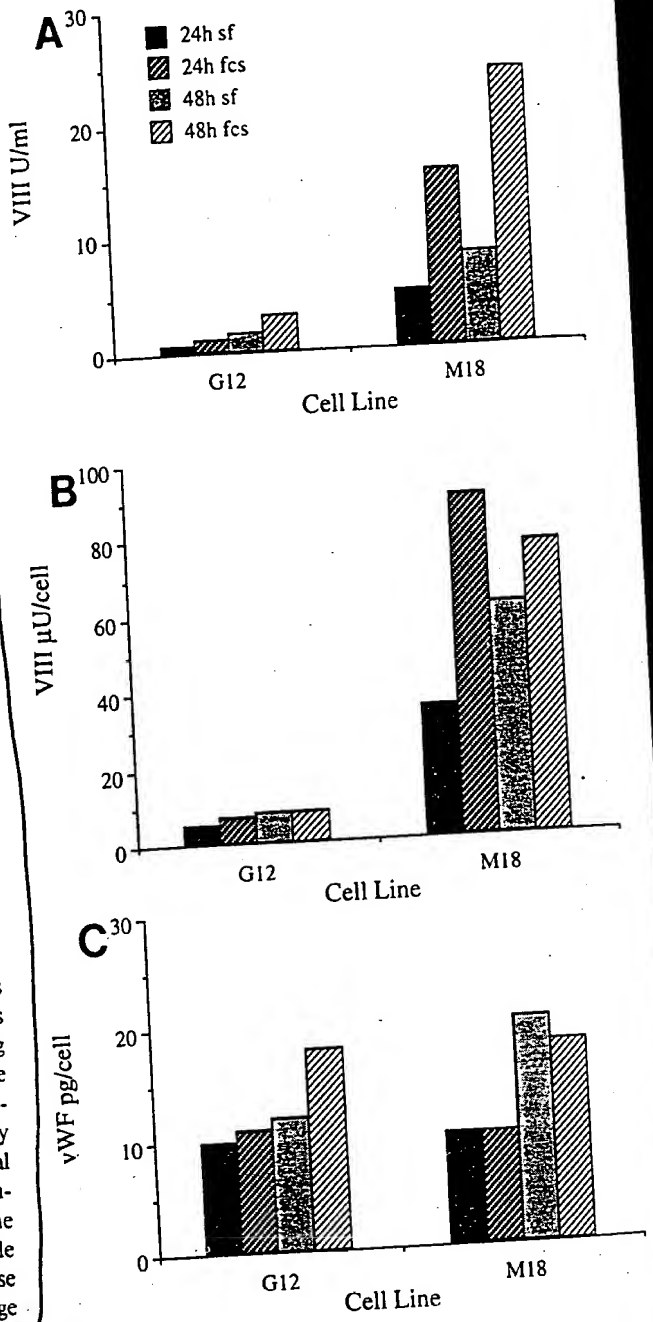

Fig 1. FVIII and vWF accumulation in G12 and M18 cells. Mid-logarithmic phase cells were fed with fresh 10% fetal bovine serum-containing medium (fcs) or serum-free medium (sf) at 0 hours. After 24 and 48 hours samples were harvested for FVIII activity assay using the FXa generation assay (A and B) and for vWF antigen using a vWF-specific ELISA (C). At the time of harvest, cells were trypsinized and counted. (A) Volumetric productivities (units per milliliter); (B and C) cellular productivities (microunits or picograms per cell).

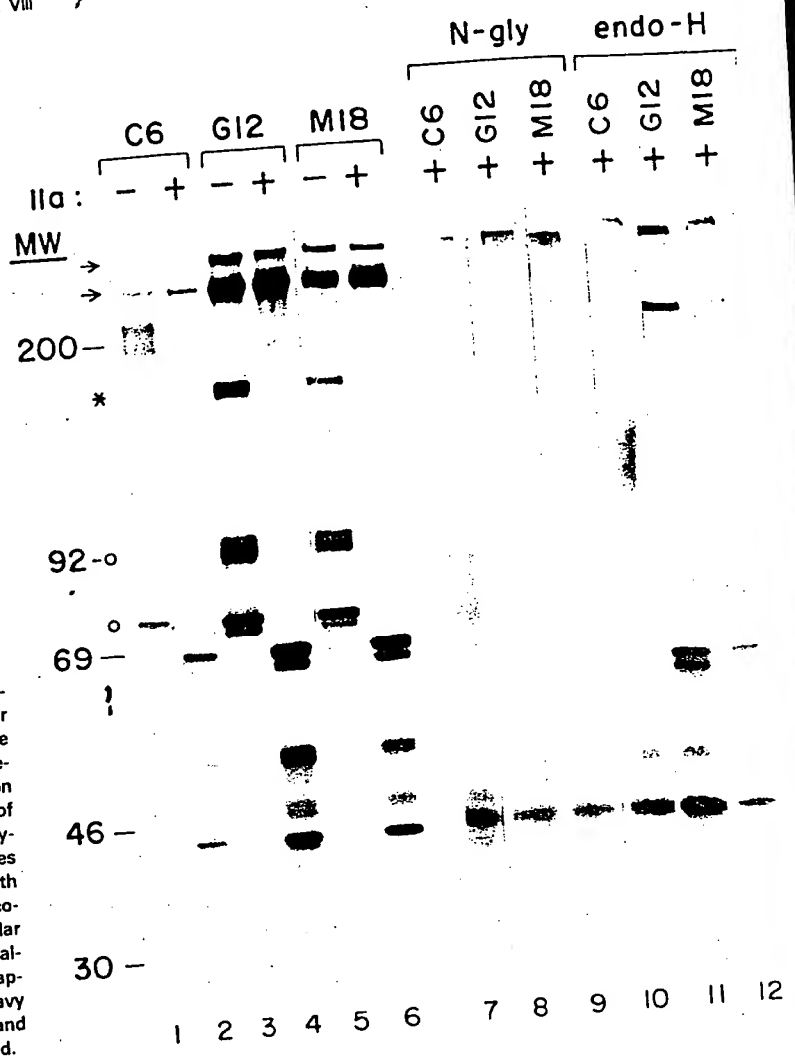

Fig 2. Comparison of LA-VIII polypeptide structure with wildtype FVIII. Cells expressing either wildtype FVIII (C6)[19] or LA-VIII (G12 and M18) were labeled with ^{35}S-methionine and conditioned medium harvested for analysis by immunoprecipitation with anti-FVIII MoAb F8[20] and electrophoresis of the immunoprecipitates on reducing SDS-polyacrylamide gels. Aliquots of the immunoprecipitates were analyzed before (−) and after treatment with thrombin (+), N-glycanase (N-gly), or endoglycosidase H (endo-H) as indicated. The molecular weight markers are shown on the left. For this analysis lanes from C6 were exposed to film for approximately a 10-fold greater time. LA-VIII heavy and light chains (O), single chain LA-VIII (*), and pro-vWF and mature vWF (arrows) are indicated.

with those of wildtype FVIII. In addition, wildtype FVIII contained a heterogeneous smear at approximately 100 Kd representing the B domain, which was absent in LA-VIII. Digestion with N-glycanase and endoglycosidase H showed that LA-VIII and wildtype VIII exhibit the same mobility shifts for the light chain 73-Kd derived fragment and the 50-Kd heavy chain derived fragment (Fig 2, lanes 7 through 12). No mobility shift was observed with the 43-Kd derived fragment, consistent with the absence of N-linked oligosaccharides on this peptide.[5] These results show that the utilization and complexity of N-linked glycosylation sites in the 50-, 43-, and 73-Kd polypeptides are similar for LA-VIII and wildtype recombinant FVIII.

Biochemical analysis of LA-VIII. LA-VIII was purified by MoAb affinity chromatography as described in Materials and Methods. The specific activity of LA-VIII was determined using an FXa generation assay and by the ability to clot FVIII-deficient plasma. The values determined were well within the range obtained for wildtype recombinant human FVIII (approximately 3,000 U/mg by Coatest assay and 2,700 U/mg by clotting assay using FVIII-deficient plasma). Thrombin activation of LA-VIII increased clotting activity approximately 30 to 100-fold, similar to that observed for wildtype recombinant FVIII (data not shown). SDS-PAGE showed that, in contrast to wildtype recombinant FVIII that exhibited the 80-Kd light chain and a heterogeneous heavy chain extending up to 200 Kd (Fig 3A, lane 1), LA-VIII was composed of three prominent species representing the 80-Kd light chain, a 92-Kd heavy chain, and a 170-Kd single chain (Fig 3A, lane 10). Upon thrombin digestion for increasing periods of time, the heavy chains of both LA-VIII and wildtype FVIII were cleaved at the same rate where maximal cleavage occurred after 1 minute. Densitometry of the thrombin cleavage products demonstrated similar kinetics of appearance of the 50- and 43-Kd heavy chain-derived polypeptides (Fig 3C). In addition, the appearance and disappearance of a 90-Kd heavy chain intermediate was similar for both molecules (Fig 3A). The difference between the 92-Kd and 90-Kd heavy-chain species for LA-VIII is likely caused by the B-domain-derived sequence (residue 741 to 1648) present on the 92-Kd species and absent on the 90-Kd thrombin cleaved species. The LA-VIII light chain was 65% cleaved after 10 seconds while wildtype recombinant FVIII required approximately 1 minute for 65% cleavage (Fig 3, A and C). Addition of human

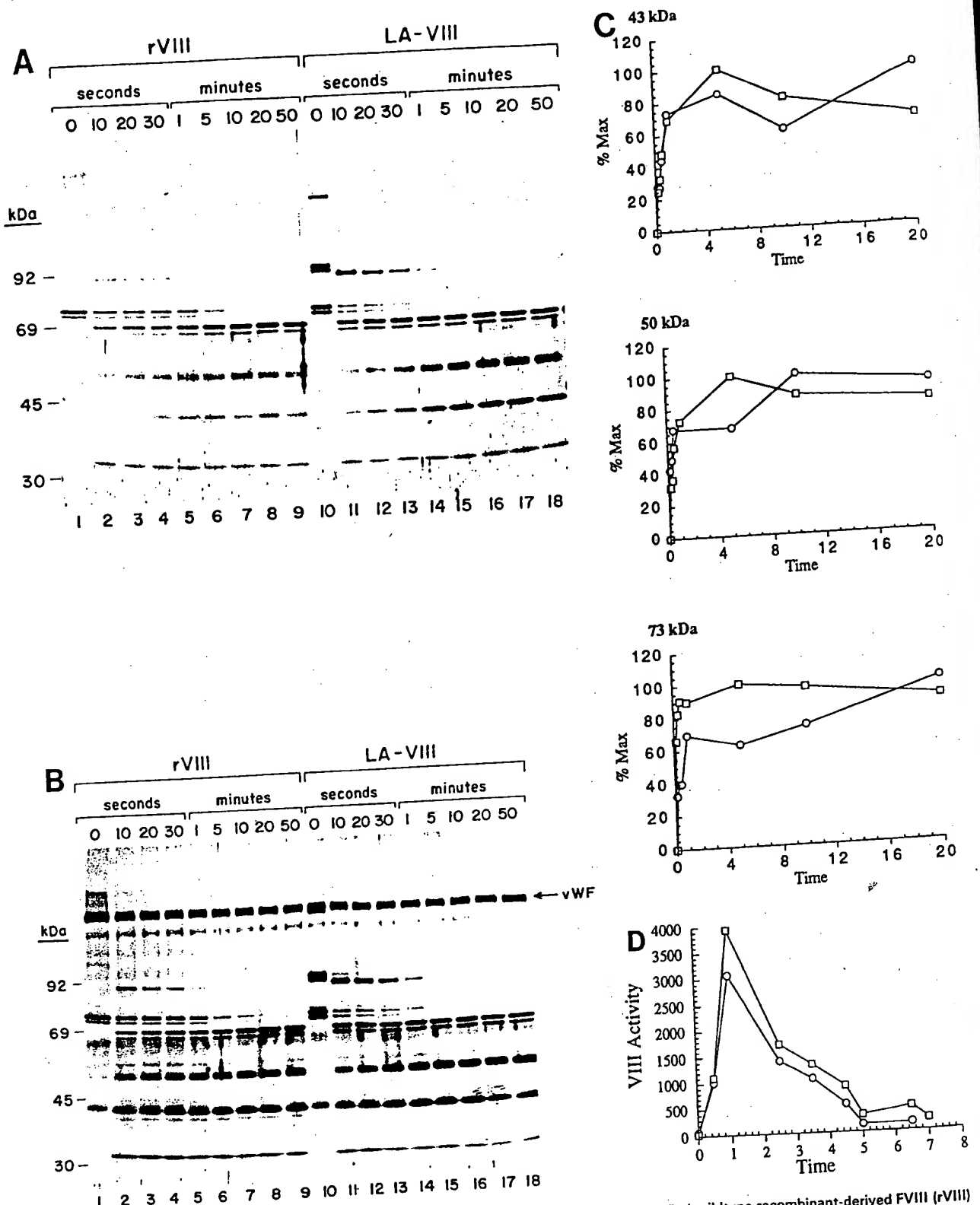

Fig 3. Thrombin cleavage and activation of wildtype recombinant FVIII and LA-VIII. Purified wildtype recombinant-derived FVIII (rVIII) or LA-VIII (each at 10 μg/mL) were treated with thrombin (0.5 μg/mL) for increasing periods of time indicated and aliquots were taken for analysis by SDS-PAGE and silver staining (A and B) and for clotting activity assay (D). For (B) the FVIII samples were preincubated with vWF (30 μg/mL) for 20 minutes at room temperature before thrombin treatment. Lanes 1 and 10 represent untreated rVIII and LA-VIII, respectively. The migration of vWF is indicated (B). Molecular weight standards are indicated on the left. The rates of appearance (percent of maximal cleavage) of the 50-, 43-, and 73-Kd fragments from rVIII (○) and LA-VIII (□) in (A) were determined by laser densitometry (C).

vWF before thrombin digestion did not alter the rate of cleavage of either the heavy chain or light chain of wildtype recombinant FVIII (Fig 3B, lanes 2 through 9) or LA-VIII (Fig 3B. lanes 11 through 18). Chromatography on CL-4B Sepharose showed that both wildtype and LA-VIII effectively bound vWF under these conditions (data not shown).

Because the rate of light chain cleavage of LA-VIII was greater than wildtype recombinant FVIII, the thrombin activation of procoagulant activity was determined by a clotting assay using FVIII-deficient plasma. Both wildtype recombinant and LA-VIII exhibited similar rapid activation of procoagulant activity with a peak after 1 minute, coincident with maximal cleavage of the heavy chain (Fig 3D). In addition, both wildtype and LA-VIII exhibited a similar rate of decay of procoagulant activity. Addition of vWF did not affect the rate of activation or inactivation of wildtype FVIII or LA-VIII (data not shown).

Immunologic characterization of LA-VIII. A neonatal mouse tolerance model was used to evaluate the immunologic similarity of LA-VIII and wildtype FVIII. This model takes advantage of the finding that administration of large quantities of a foreign antigen (tolerogen) to a newborn mouse renders the mouse specifically nonresponsive to that foreign antigen. The ability for a related antigen to induce an antibody response indicates that the immune system of the tolerized mouse recognizes distinct epitopes not present in the original tolerogen. Preliminary studies indicated that a single 25-μg dose of plasma-derived or recombinant-derived wildtype FVIII administered by intraperitoneal injection to neonatal Balb/CBy mice within 30 hours of birth resulted in the induction of a state of antigen-specific nonresponsiveness in 89% (48 of 54) treated animals. Mice tolerized to recombinant-derived FVIII were nonresponsive to subsequent challenge with recombinant- or plasma-derived FVIII. Similarly, mice tolerized with plasma-derived FVIII were nonresponsive on subsequent challenge with plasma- or recombinant-derived FVIII. Of 43 mice that received recombinant-derived FVIII challenge, only 1 responded with antitest antigen (a positive response is defined by titer >100). Immunocompetence of experimental mice was scored by measuring the response to an unrelated immunogen, antigen KLH. All mice mounted significant response to this control immunogen, demonstrating full competence, and that any observed reduction in response to the test antigen was specific in nature. In contrast, all sham-tolerized mice mounted significant response to both the control antigen (KLH) and to test antigen (FVIII) (data not shown). These results support immunologic similarity of wildtype plasma-derived and recombinant-derived FVIII in the model system.

We then evaluated whether the tolerance developed to wildtype FVIII could be broken with a challenge of LA-VIII using the protocol described Table 1, section A. Newborn mice were either sham tolerized (groups A and B) or tolerized with wildtype recombinant VIII (rVIII) (groups C and D) or LA-VIII (group E). Ten days later the mice were challenged with either wildtype rVIII (groups A, D, and E) or LA-VIII (groups B and C). The data in Table 1, section B, show the amount of specific anti-FVIII Ig (IgG) detected by titration ELISA using either wildtype rVIII or LA-VIII. At day 16, 1 of 14 mice theoretically tolerized to rVIII showed borderline reactivity against both wildtype rVIII and LA-VIII (from group C, Table 1, section B). The antibody titers measured at day 32 were averaged both excluding and including the mouse showing significant anti-FVIII response (Table 1. section B). At day 32, significant anti-FVIII antibody was detected in all sham-tolerized animals that were challenged with either rVIII or LA-VIII (groups A and B). Significantly. 10- to 20-fold reduced titers were observed in mice that were tolerized with rVIII and challenged with rVIII or LA-VIII (groups C and D). The nonresponsiveness to rVIII and LA-VIII was specific because no difference in antibody response toward KLH was detected between the sham-tolerized and rVIII- or LA-VIII tolerized mice. In contrast, mice that were tolerized to LA-VIII and challenged with wildtype rVIII (group E) developed a significant anti-FVIII response after 32 days. The anti-FVIII antibodies in the LA-VIII tolerized mice were reactive with LA-VIII as well as wildtype rVIII. These results show that tolerance to wildtype rVIII could not be broken by either wildtype rVIII or LA-VIII challenge. In contrast, tolerization to LA-VIII could be broken by wildtype rVIII challenge.

To further characterize the response detected by ELISA. the day 32 murine sera were used to probe Western blots in which either wildtype FVIII or LA-VIII were electrophoresed on an SDS-polyacrylamide gel and then transferred to nitrocellulose. The filters were probed with sera from the challenged mice as well as a rabbit anti-human FVIII polyclonal antibody (as positive control). The control anti-FVIII antibody showed strong reactivity with both wildtype and LA-VIII FVIII whereas no reaction was detected with the 9 mice sera tested (data not shown), suggesting that the specificity of affinity of anti-FVIII response detected by ELISA in the serum was very low or that the antibody generated did not react by Western analysis. In conclusion, these experiments show that the mouse immune system could not detect any different epitopes between LA-VIII and wildtype FVIII.

In vivo efficacy of LA-VIII. The in vivo efficacy of purified preparations wildtype FVIII and LA-VIII were tested by infusion into two severely affected, congenitally FVIII-deficient animals as described.[23] LA-VIII was administered on day 1. followed by an identical study performed 2 days later with the wildtype FVIII. Blood samples were obtained pre- and post-FVIII infusion at various times. Coagulation testing performed on each sample included FVIII functional activity as measured in a one-stage clotting assay, fibrinogen levels. and a full blood count including platelets. Bleeding times were determined, after bleeding was induced in lightly anesthesized animals, by severing the apex of the nail cuticle using a guillotine device. The length of time for this induced bleeding to be arrested is the cuticle bleeding time (CBT). This stringent model for FVIII activity is based on the reproducible long bleeding time that occurs and for which cessation requires infusion of functional FVIII.[24] For both the wildtype and LA-VIII FVIII, the infusion was tolerated without any observable side effects. In the first study, performed in a severely affected homozygous female, the FVIII activity and antigen survival curves for both preparations were very similar and in line with the anticipated t1/2 of 12 to 13 hours for

16 20

16 20

16 20

5 7 8

FVIII (rVIII)
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plasma-derived FVIII (Fig 4, A and B). In the second study, performed in a severely affected male, very similar results were obtained with regard to the recovery and survival of functional FVIII activity for both preparations (data not shown). FVIII antigen levels were not measured in the second study. Plasma samples obtained in the first study 1 hour after infusion of both the wildtype and LA-VIII were fractionated by Sepharose-4B chromatography and analyzed for the presence of FVIII and vWF antigen. Canine vWF antigen eluted between fractions 7 and 15 for animals that received either LA-VIII or wildtype FVIII. Analysis of FVIII activity showed that both preparations had a significant and similar amount of FVIII antigen co-eluting with vWF, indicating that the vWF binding characteristics for wildtype FVIII and LA-VIII were similar (Fig 4C). CBTs were performed before and at 30 minutes and 2 hours (study 1), and 30 minutes, 1 hour, and 2 hours (study 2) after the infusion of both preparations of FVIII. In each case, the cuticle bleeding time was corrected following the infusion of LA-VIII. The FVIII levels at the time of performing the CBTs in study 1 were 44% (30 minutes) and 40% (2 hours) of the normal canine plasma level. In the case of the second study, the levels were 146% (30 minutes), 136% (1 hour), and 127% (2 hours). In the case of the first study, the CBT was corrected 30 minutes after the infusion of wildtype FVIII but not at 2 hours. The FVIII level at these times was 55% and 47%, respectively. In the second study, the CBTs were corrected at 30 minutes, 1 hour, and 2 hours after the infusion of wildtype FVIII when the levels were 106%, 92%, and 109%, respectively. Based on the stringent requirement for FVIII for correction of the CBT in this model system, the results show that LA-VIII is functional in vivo.

DISCUSSION

The present study was undertaken to evaluate the biochemical, immunologic, and in vivo functional properties of a B-domain–deleted mutant of FVIII, designated LA-VIII. LA-VIII was expressed at approximately 10- to 20-fold greater levels in CHO cells compared with wildtype FVIII when similar vectors, cells, and methods were used. The increased expression resulted from increased levels of LA-VIII mRNA (data not shown) and increased secretion efficiency. The improved secretion efficiency correlated with reduced interaction with the glucose regulated protein of 78 Kd (GRP78 or BiP).[12,27,28] The expression levels attained, greater than 20 U/mL, were an order of magnitude greater than previous reports on B-domain–deleted FVIII.[7,10,11] One posttranslational modification that may have become limiting for biosynthesis of functional FVIII at these high expression levels was sulfation of tyrosine. However, even at this high expression level we have previously shown that tyrosine sulfation was efficient.[29] Biochemically, LA-VIII was similar to wildtype FVIII with respect to specific activity in clotting assays and FXa generation assays and also exhibited similar thrombin activation coefficients.

The single most significant difference between LA-VIII and wildtype FVIII was that the light chain of LA-VIII was more susceptible to thrombin cleavage. No difference was detected in the rate of heavy chain cleavage by thrombin. In addition,

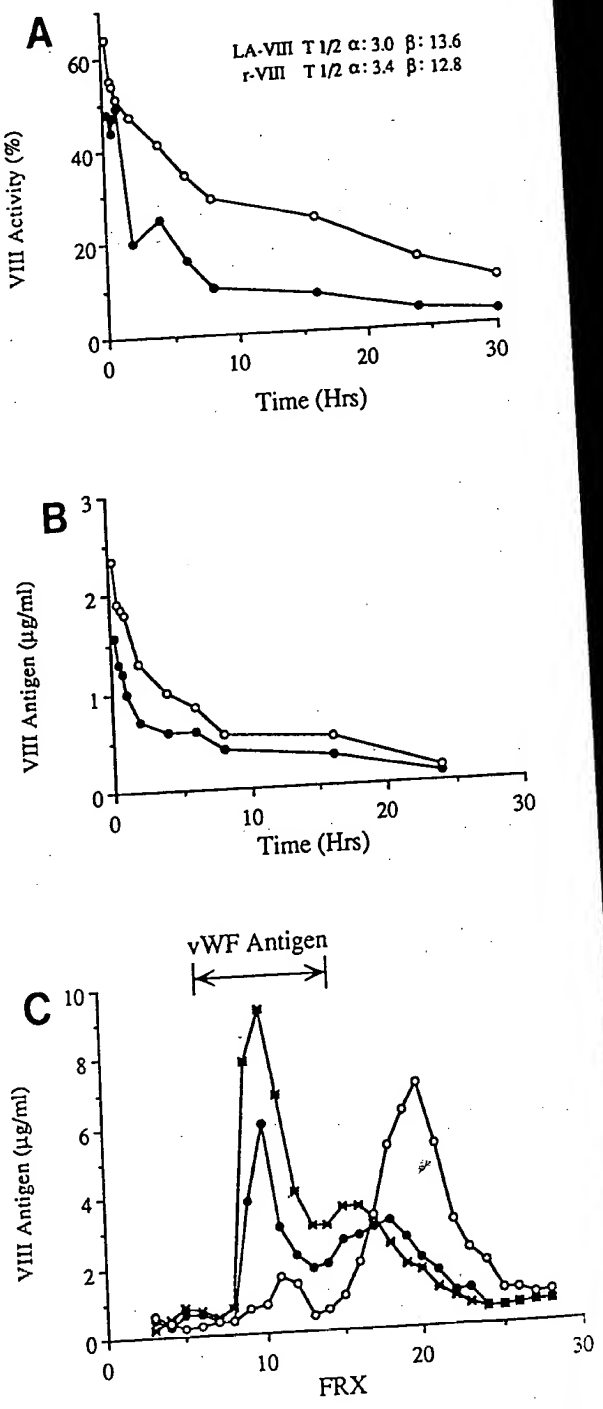

Fig 4. Recovery and vWF binding of infused wildtype FVIII and LA-VIII. After infusion of LA-VIII (○) and wildtype recombinant factor VIII (●) into the hemophilic dog, plasma samples were taken at the times indicated and FVIII functional activity (A) and antigen (B) levels were determined as described in Materials and Methods. The T1/2 for the α-phase and β-phase of elimination are indicated for both LA-VIII and rVIII. (C) Plasma samples taken at 1 hour postinfusion of wildtype FVIII (X) or LA-VIII (●) were analyzed by chromatography on Sepharose-4B and eluting fractions were assayed for FVIII antigen and vWF antigen as described in Materials and Methods. Wildtype FVIII was processed in a similar manner without infusion into a dog (○).

there was no difference in the rate of activation of procoagulant activity. For both LA-VIII and wildtype FVIII, peak activation correlated with maximal cleavage of the heavy chain. This is consistent with previous results showing that procoagulant activity is associated with cleavage at residue 372 and does not require cleavage of the light chain.[30] These results are different than those of Eaton et al[10] where a B-domain deletion (residues 797 through 1562) molecule was shown to exhibit increased sensitivity to thrombin activation compared with wildtype and those of Meulien et al[11] where a B-domain deletion (residues 771 through 1666) molecule was activated to a greater extent than wildtype FVIII. However, in both of these studies the rate of thrombin cleavage of the B-domain–deleted molecule was not compared with wildtype FVIII. Although it is likely that different deletions will exhibit slightly different properties, the properties of LA-VIII described here closely resemble wildtype FVIII.

Previously, it was shown that vWF inhibited activity of *Bothrops jararacussu* venom activated porcine FVIII, presumably by binding to the intact FVIII light chain.[30] It was proposed that cleavage of the FVIII light chain was required to release vWF and elicit FVIII procoagulant activity. In addition, vWF promoted thrombin cleavage of the FVIII light chain.[31] Therefore, we examined the effect of vWF on the cleavage and activation of human wildtype recombinant FVIII and LA-VIII. Addition of vWF did not affect either the procoagulant activity or the rate of light or heavy chain cleavage of either wildtype FVIII or LA-VIII. The difference between our results and those of Hill-Eubanks and Lollar may be attributed to the different species of FVIII being studied (human *v* porcine) and different assay systems (clotting assay and FXa generation assay).

Because of the increased susceptibility of the light chain to thrombin cleavage, we asked whether the molecule was safe and efficacious on infusion into a hemophilic dog. The results of two studies in two hemophilic dogs showed that the half-life of both antigen and activity of LA-VIII was similar to wildtype recombinant FVIII. In vivo, LA-VIII was also complexed with canine vWF to a similar degree as wildtype recombinant FVIII. In addition, LA-VIII was well tolerated and did not affect any hematologic parameters studied, including fibrinogen levels or platelet counts. LA-VIII was capable of correcting the CBT, approximately similar to wildtype recombinant FVIII. The results of these studies indicate that LA-VIII is functional in this animal model.

A neonatal murine tolerance model was used to identify potential new or cryptic epitopes within LA-VIII. Administration of relatively large quantities of a foreign antigen to a newborn animal before the occurrence of 'self–nonself' discrimination events within the immature immune system can lead to a state of antigen-specific immunologic nonresponsiveness toward the foreign antigen.[32,33] This response renders the animals specifically nonresponsive to the foreign antigen (tolerogen), which has been misidentified as a normal component of the protein repertoire of the tolerized animal. Specific nonresponsiveness to the tolerogen can be broken only by the administration of a similar, but distinct, antigen (xenoantigen) that shares many, but not all, epitopes present in the tolerogen. Response to these dissimilar epitopes on the challenge of xenoantigen results in the development of antibodies that may cross-react with the original tolerogen. If the animal develops no antibodies to the test antigen, the test antigen and the tolerogen may be considered immunologically identical.

These observations were used to design a neonatal murine model system of tolerance induction to evaluate immunologic equivalence of plasma-derived FVIII, wildtype recombinant-derived FVIII, and LA-VIII. Control studies demonstrated that tolerance could be induced in almost 90% of mice by a single infusion of either recombinant-derived or plasma-derived FVIII into neonatal mice. Mice tolerized to plasma-derived FVIII also exhibited tolerance to recombinant-derived FVIII and vice versa. Thus, this analysis could not detect any immunologic differences between plasma-derived and recombinant-derived FVIII. Upon challenge with wildtype recombinant-derived FVIII, mice tolerized to LA-VIII mounted a vigorous antibody response to both wildtype recombinant-derived FVIII and LA-VIII. The response to both immunogen and tolerogen is characteristic of termination of the tolerant state.[32,34] In contrast, upon LA-VIII challenge of mice tolerized to wildtype FVIII, only one of nine mice exhibited significant detectable antibodies that reacted with wildtype as well as LA-VIII. However, the antibody response could not be confirmed by Western immunoblot analysis, suggesting the antibodies generated may be of low affinity and/or titer to permit their detection by these procedures. We speculate that the response in the 1 of 9 mice was due to failure to tolerize the single responding mouse. The significant lack of response by the majority of the mice indicated that LA-VIII is immunologically indistinguishable from wildtype FVIII. Previous immunologic characterization of another FVIII B-domain deletion molecule showed a specific immunologic response to the fusion junction peptide. In this case, the polyclonal antibody generated was characterized by immunoadsorption and cross-reactivity to peptides and wildtype FVIII.[35] Our approach of neonatal tolerance induction should be more sensitive to identify any significant epitopes. Thus, we may have expected to obtain an antibody response to the fusion joint of the deletion molecule. However, LA-VIII contains a potential N-linked glycosylation site at the fusion junction and may protect the protein from an antibody response. At present we do not know if the N-linked site is actually occupied with oligosaccharides. In addition to the ability to compare the immunologic properties of different derivatives of FVIII, the tolerance induction for FVIII in neonatal mice described here should provide a useful approach to derive mice that are tolerant to human FVIII for use as recipients for FVIII gene therapy to circumvent potential complications of antibody development to human proteins.[36]

The results reported here suggest that B-domain–deleted FVIII (LA-VIII) is safe, efficacious, and immunologically similar to wildtype FVIII. To date a physiologically significant function cannot be attributed to the B domain. The particular advantages of B-domain–deleted FVIII for therapeutic use include its greater level of expression and significantly reduced heterogeneity. Thus, the cost for production should be significantly less than wildtype FVIII, thereby possibly permit-

ting prophylactic treatment. Prophylactic use of FVIII may significantly reduce orthopedic complications.[37] In addition, the cDNA encoding B-domain–deleted FVIII is sufficiently small to allow incorporation into retroviral vectors for gene therapy. Previously it was shown that significant levels of LA-VIII could be expressed using a retroviral vector.[38] The results presented here suggest that B-domain–deleted FVIII may be efficacious for replacement therapy and/or gene therapy for hemophilia A.

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A large region (≈95 kDa) of human factor VIII is dispensable for *in vitro* procoagulant activity

(*in vitro* mutagenesis/coagulation/evolution)

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ABSTRACT Factor VIII (antihemophilic factor) is a high molecular weight plasma glycoprotein that participates in the blood clotting cascade. The recent cloning and sequence analysis of the cDNA encoding human factor VIII revealed an obvious domain structure for the protein, which can be represented as A1-A2-B-A3-C1-C2. We now report the DNA sequence analysis of porcine exons encoding the entire B domain and part of the A2 and A3 domains. We found an unusually high degree of porcine–human amino acid sequence divergence in the B region compared with the limited sequence available for other regions of the porcine factor VIII molecule. In addition to sequence divergence, there are numerous gaps in the porcine B domain totalling over 200 amino acids. Recombinant DNA techniques were used to effect the removal of large segments of DNA encoding the B domain from the full-length human factor VIII cDNA. These constructs directed the synthesis of biologically active factor VIII when introduced into mammalian cells despite the deletion of up to 38% of the factor VIII molecule.

Hemophilia A is a bleeding disorder resulting from a deficiency or abnormality of the blood clotting protein, factor VIII (for review, see ref. 1). Factor VIII functions in the blood clotting cascade as the cofactor for factor IXa proteolytic activation of factor X. The blood clotting pathway in which factor VIII participates eventually results in the proteolytic cleavage of fibrinogen to form insoluble fibrin polymers. *In vivo*, fibrin deposition in conjunction with platelet aggregation act to curtail blood loss from a damaged vessel.

The cofactor activity of factor VIII acts to increase the V_{max} of the factor IXa-dependent activation of factor X by at least 4 orders of magnitude. Factor VIII does not function proteolytically in this reaction but can itself be proteolytically activated by other coagulation enzymes such as factor X or thrombin. Neither the mechanism of factor VIII activation nor the nature of its cofactor activity is well understood.

Recently the entire human factor VIII gene, spanning over 185 kilobases (kb) of the X chromosome, and full-length cDNA have been cloned (2–5). The DNA sequence of the cDNA and NH_2-terminal amino acid sequence analysis of the plasma protein shows factor VIII to be synthesized as a single-chain precursor of 2351 amino acids from which a 19-amino acid "signal sequence" is cleaved during translation. A computer-aided search for factor VIII intramolecular homologies revealed three distinct structural domains, including a triplicated region (A domain) of ≈330 amino acids, a duplicated region (C domain) of ≈150 amino acids, and a unique region (B domain) of ≈980 amino acids. These domains are arranged in the order A1-A2-B-A3-C1-C2 (see Fig. 1) as described by Vehar *et al.* (5). The B domain is essentially delimited by residues 740 and 1689 that are sites for proteolytic activation of the molecule and, therefore, appears like an unusually large activation peptide. This region is extremely rich in potential asparagine-linked glycosylation sites, containing 20 of the 25 sites found in factor VIII.

In this report, we present DNA sequence analysis that demonstrates a very high degree of divergence between porcine and human B domains of the factor VIII gene. In addition, removal of the majority of DNA encoding the B domain from a full-length human factor VIII cDNA is not detrimental to the synthesis of biologically active factor VIII in a mammalian host cell.

MATERIALS AND METHODS

Plasmid Constructions. To construct pDGR-2, 10 μg of the plasmid pACE, a pSP64 (Promega Biotec, Madison, WI) derivative, containing nucleotides 562–7269 of human factor VIII cDNA (nucleotide 1 is the A of the ATG initiator methionine codon) was subjected to partial *Bam*HI digestion in 100 μl containing 50 mM Tris·HCl (pH 8.0), 50 mM $MgCl_2$, and 2.4 units of *Bam*HI (New England Biolabs) for 30 min at 37°C. The reaction was terminated by the addition of EDTA to 10 mM and the mixture was then extracted once with phenol and once with chloroform, ethanol-precipitated, and pelleted by centrifugation. DNA was redissolved, cleaved to completion in 50 μl using 40 units *Sac* I for 1.5 hr at 37°C. DNA was then electrophoresed through a buffered 0.6% agarose gel. An 8.1-kb fragment corresponding to the partial *Bam*HI/*Sac* I fragment of pACE lacking only the sequence corresponding to nucleotides 2992–4774 of the factor VIII sequence was purified from the gel by using the glass powder technique (6). Purified DNA was ligated with 100 pmol of the following double-stranded oligonucleotide:

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5' pCATGGACCG 3'
3' TCGAGTACCTGGCCTAG 5'
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DNA was then used to transform competent *Escherichia coli* bacteria and DNA from several ampicillin-resistant transformants was analyzed by restriction mapping to identify a plasmid harboring the desired *Sac* I/*Bam*HI deletion mutant. DNA from this plasmid was digested to completion with *Kpn* I, which cleaves the plasmid uniquely at nucleotide 1816 of the factor VIII coding sequence. This DNA was ligated with a *Kpn* I DNA fragment containing nucleotides 1–1815 of factor VIII DNA and a synthetic *Sal* I site at nucleotides −11 to −5 and then used to transform competent *E. coli* bacteria.

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Abbreviation: kb, kilobase(s).

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Plasmid DNA was isolated and oriented by restriction mapping to identify a plasmid containing the correct 5′ to 3′ orientation of the *Kpn* I insert. *Sal* I digestion, which excises the entire polypeptide coding region from the plasmid, was performed and the DNA was electrophoresed through a buffered 0.6% agarose gel. The 5.3-kb *Sal* I fragment was purified from the gel as described above. This DNA fragment was ligated with *Xho* I-cut pXMT2 DNA to give rise to plasmid pDGR-2. pXMT2 is a plasmid capable of expressing heterologous genes when introduced into mammalian cells such as the COS-1 African green monkey kidney cell line, and is a derivative of the expression vector p91023(B) (7, 8). The unique *Xho* I site of pXMT2 allows for expression of inserted cDNA from the upstream promoter. Restriction mapping of transformants identified a plasmid, pDGR-2, containing the correct 5′ to 3′ orientation of the polypeptide coding sequence relative to the direction of transcription.

pLA-2 was created by using the loop-out mutagenesis technique described by Morinaga *et al.* (9). Briefly, 1 μg each of pDGR-2 and pXMT2 was linearized by restriction endonuclease digestion with *Eco*RV and *Xho* I, respectively, denatured in 20 μl containing 0.2 M NaOH for 10 min at room temperature, and then neutralized by addition of 180 μl of 0.02 M HCl and 0.1 M Tris·HCl (pH 8.0). The DNA was then reannealed for 90 min at 68°C. The sample (40 μl) was then slowly cooled to room temperature after addition of an excess of the kinased 40-mer oligonucleotide 5′ AAAAGCAATTTAATGCCACCCCACCAGTCTTGAAACGCCA. The sample was brought to 50 μl containing 0.1 M Tris·HCl (pH 8.0), 20 mM NaCl, 2 mM $MgCl_2$, 500 μM ATP, 100 μM deoxynucleotide triphosphate, Klenow fragment of *E. coli* DNA polymerase (4 units/μl) and T4 ligase (400 units/μl). The reaction mixture was incubated for 16 hr at 15°C and then the reaction was terminated by phenol/chloroform extraction and nucleic acids were ethanol-precipitated. DNA was then used to transform competent *E. coli* bacteria; ampicillin-resistant colonies were screened by using the ^{32}P-labeled 14-mer 5′ TGCCACCCCACCAG. Restriction mapping identified a plasmid, pLA-2, containing the desired deletion.

DNA Sequencing. The porcine DNA sequence was derived from factor VIII genomic clones containing exons corresponding to human exons 14 and 15. DNA sequence analysis was obtained from numerous overlapping fragments subcloned into M13 and subjected to the dideoxy-sequencing technique as described by Sanger *et al.* (10).

COS Cell Transfections and Analysis of Procoagulant Activity. COS cells (11) were transfected with 8 μg of each plasmid per 10-cm dish in 4 ml of DEAE-dextran in Dulbecco's modified Eagle's conditioned medium as described (7). Forty-eight hours post-transfection, 10 ml of fresh medium containing 10% fetal bovine serum was applied and samples were taken for factor VIII activity assay 24 hr later as described by Toole *et al.* (2).

Immunoprecipitation Analysis. Cells were transfected and, 48 hr post-transfection, they were labeled with [^{35}S]methionine (0.5 mCi/ml; specific activity, >800 Ci/mmol; 1 Ci = 37

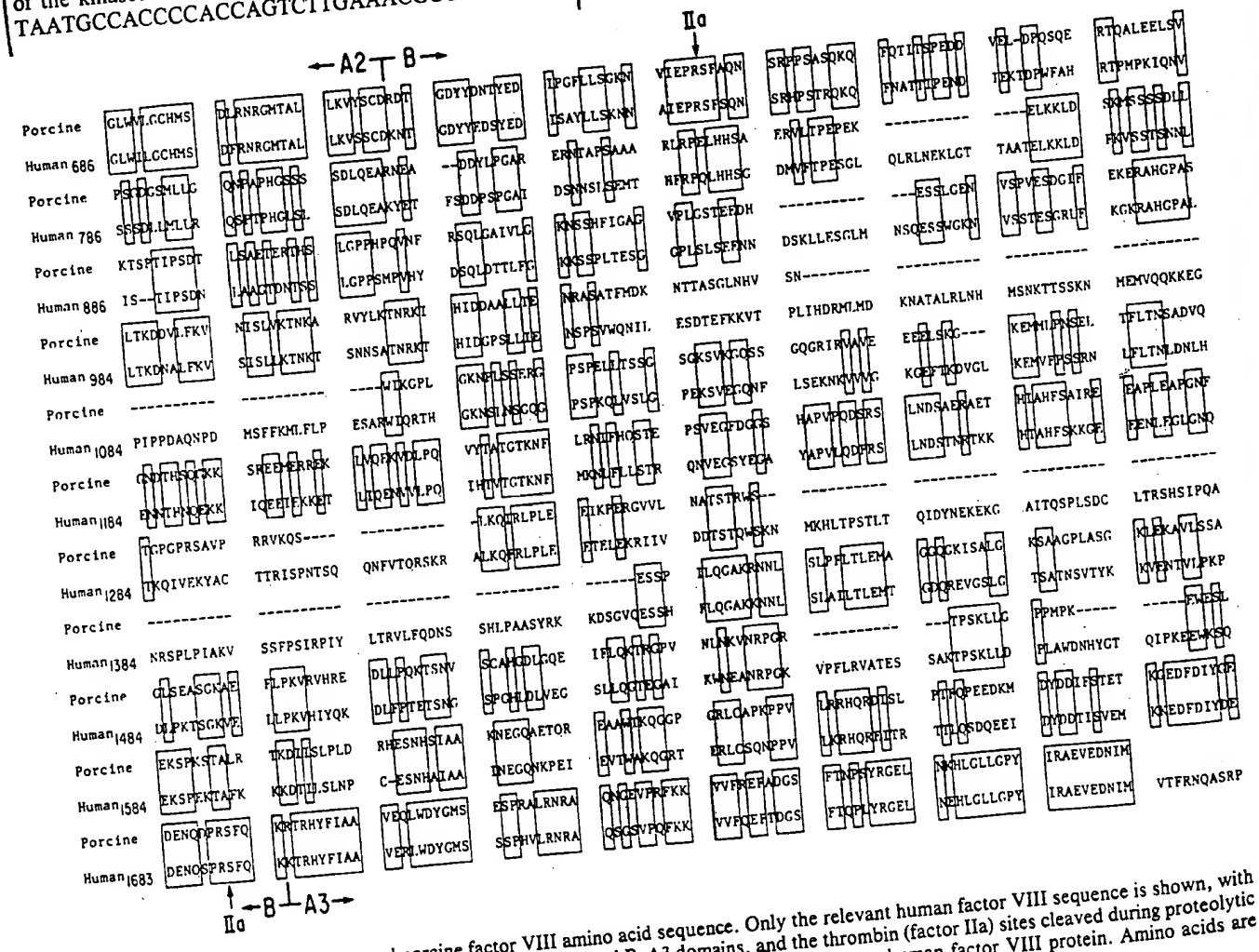

FIG. 1. Comparison of human and porcine factor VIII amino acid sequence. Only the relevant human factor VIII sequence is shown, with homologous amino acids boxed. The boundaries of the A2–B and B–A3 domains, and the thrombin (factor IIa) sites cleaved during proteolytic activation are identified. The subscript numbers refer to the amino acid number of the mature human factor VIII protein. Amino acids are designated by the single-letter code.

GBq; New England Nuclear) in the presence of serum-free medium. Six hours later, equal aliquots of conditioned medium were taken for immunoprecipitation with a monoclonal antibody (Hybritech, San Diego, CA) that recognizes the light chain (76 kDa) of factor VIII, using rabbit anti-mouse IgG as the precipitating antibody. Immunoprecipitates were analyzed by $NaDodSO_4$ reducing gel electrophoresis as described by Kaufman and Sharp (12).

RESULTS

The sequence of the human factor VIII cDNA has been reported (2, 4). In addition to the human clones, we isolated a porcine factor VIII genomic clone homologous to the human factor VIII recombinant phage containing exon 14, a 3106-base-pair exon encoding the entire B domain. We subsequently walked 3′ in the porcine genomic library and isolated a clone homologous to human exon 15. DNA sequencing of both porcine exons was completed and the porcine factor VIII amino acid sequence was deduced. A comparison of the corresponding porcine and human factor VIII amino acid sequences is shown in Fig. 1. Remarkably, there exist several substantial deletions totaling >200 amino acids in the porcine B domain relative to the human. In addition, there is a marked diminution of homology within the B domain compared to either that observed in the A2–A3 regions shown in Fig. 1 or the limited regions derived from NH_2-terminal amino acid sequencing of porcine factor VIII (2). Specifically, there is ≈50% homology, excluding the deletions, in the B domain, whereas the known amino acid sequence elsewhere in the factor VIII molecule exhibits 80–85% homology.

Considering this striking divergence and since porcine factor VIII corrects the hemophilia A coagulation deficiency *in vivo* (13) and *in vitro*, we postulated that the B domain is not involved in procoagulant activity *per se*. To examine this we used either restriction enzymes or site directed loop-out mutagenesis to remove a segment of human factor VIII cDNA encoding the B domain, and we tested the protein encoded by the new constructs for procoagulant activity. We used restriction enzymes *Sac* I and *Bam*HI and an oligonucleotide linker to effect the removal of cDNA encoding 581 amino acids (residues 1001–1581) while maintaining the translational reading frame. We also used a 40-mer oligonucleotide to loop out cDNA encoding 880 amino acids following the procedure described in Morinaga *et al.* (9). This larger deletion begins 19 amino acids after the thrombin cleavage site at residue 740 and ends 50 amino acids before the thrombin cleavage site at residue 1689. The positions of the deletions in the factor VIII molecule are schematically shown in Fig. 2. The smaller deletion removes 13 potential asparagine-linked glycosylation sites; the larger deletion removes 18 of the 20 total sites in the B domain. Factor VIII molecules missing 581 or 880 amino acids are encoded by plasmids pDGR-2 and pLA-2, respectively.

The factor VIII cDNAs deficient in B-domain polypeptide were placed into the mammalian cell expression vector pXMT-2, a derivative of p91023(B) (7, 8). We have previously demonstrated that a similar vector (pCVSVL) directs the synthesis of biologically active factor VIII when the DNA is introduced by transfection into the African green monkey cell line COS-1 (2).

The expression plasmids containing the modified factor VIII cDNAs and the full-length cDNA, pXMT-VIII, were introduced into COS-1 cells via the DEAE-dextran transfection protocol (14). Conditioned medium was harvested 48 hr post-transfection and assayed for factor VIII activity as described by Toole *et al.* (2). The results of the experiment are summarized in Table 1. Surprisingly, both plasmids containing the deleted factor VIII cDNAs yielded procoagulant activity and, moreover, the activity was greater than

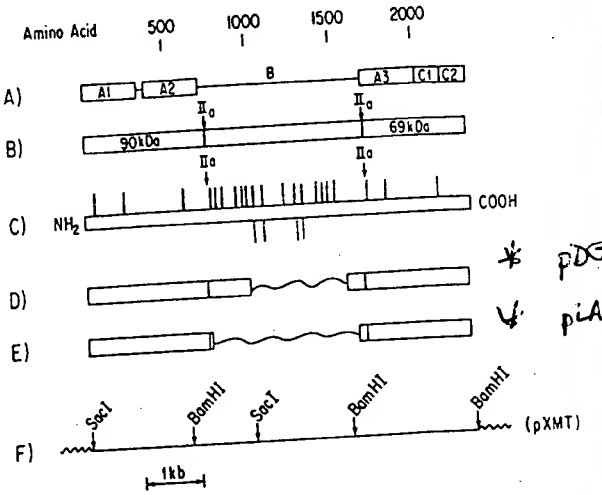

FIG. 2. Structural features of factor VIII are diagrammatically presented. Line A shows the relative positions of the domains as described by Vehar *et al.* (5). Line B shows the position of the sites cleaved by thrombin (IIa) during proteolytic activation of the molecule. Line C shows the relative positions of potential NH_2-linked carbohydrate addition sites (Asn-X-Ser or Thr). Lines D and E depict, by wavy lines, the regions deleted from factor VIII. The molecules diagrammed in lines D and E are encoded by plasmids pDGR-2 and pLA-2, respectively. Line F is a restriction map showing the relative location of *Bam*HI and *Sac* I sites in human factor VIII cDNA.

that obtained from wild-type cDNA. One possible explanation for this increased activity level is that these molecules are in a conformation that mimics to some extent the proteolytically activated form of the protein. To examine this, we determined the thrombin activatability, as determined by a factor VIII deficient plasma assay, of the modified factor VIII molecules in the conditioned medium. If the abridged factor VIII molecules were in a partially or fully activated conformation, then exposure to catalytic amounts of thrombin should result in a decreased enhancement of activity. We found that the novel forms of factor VIII had their activity enhanced to the same extent, 20- to 30-fold, as wild type after exposure to thrombin. We conclude from these data that removal of up to 880 amino acids (≈95 kDa) in a defined domain of human factor VIII does not destroy cofactor activity. Furthermore, these abridged procoagulant proteins retain their ability to be activated by thrombin.

Table 1. Expression of abridged factor VIII molecules

	Amino acids deleted	Chromogenic activity, milliunits/ml	Clotek activity	
			− IIa	+ IIa
No DNA	—	0		
pXMT-VIII	—	15.1	—	450
pDGR-2	581	114	250	5750 (23)
pLA-2	880	162	330	9240 (28)

The plasmids indicated were transfected into COS cells and 48 hr post-transfection the conditioned media were taken for assay by the Kabi Coatest factor VIII:C method (chromogenic activity) and by the one-stage activated partial thromboplastin time coagulation assay (Clotek activity) using factor VIII:C-deficient plasma. For thrombin activation, samples were pretreated 1–10 min with 0.2 units of thrombin per ml (IIa) at room temperature. Activation coefficients are given in parentheses. Activity from medium from the wild-type (pXMT-VIII) transfection was too low to directly measure Clotek activity before thrombin activation. From other experiments where the wild-type factor VIII activity was concentrated, it was demonstrated to be ≈30-fold activatable. Also, separate cotransfection experiments have demonstrated that the differences in activities are not due to differences in transfection efficiencies.

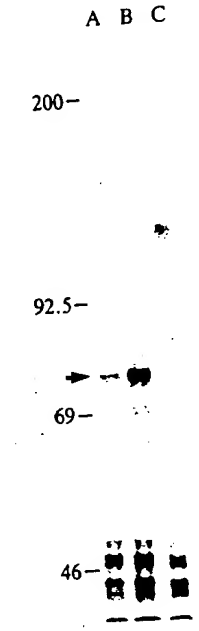

FIG. 3. Factor VIII synthesis and secretion in transfected COS cells. COS cells were transfected and [^{35}S]methionine-labeled factor VIII was detected by immunoprecipitation and autoradiography as described. Results with transfection of pXMT-VIII (lane A), pDGR-2 (lane B), and mock (no DNA; lane C) are presented. Arrow indicates the predicted 76-kDa species reactive with the anti-factor VIII monoclonal antibody. The heavy chain of factor VIII is not detected in this analysis.

We next wanted to determine the reason for the increased activity observed in the conditioned medium of pDGR-2 and pLA-2 transfectants. To do this, cells were labeled with [^{35}S]methionine and 48 hr post-transfection, conditioned medium from the cells was then immunoprecipitated using a monoclonal antibody specific for the 76-kDa light chain of human factor VIII. The precipitate was then subjected to $NaDodSO_4$/PAGE autoradiography. As shown in Fig. 3, considerably more of the 76-kDa polypeptide is precipitated from the medium of cells transfected with pDGR-2 compared to pXMT-VIII (wild type). The increase in immunoprecipitable radiolabeled factor VIII approximately reflects the differences in activity observed in the cultures (≈10-fold). Thus, the removal of polypeptide from the B domain of human factor VIII results in procoagulant proteins having a specific activity similar to the natural protein. However, these abridged forms of factor VIII accumulate to higher levels, compared to wild type, in medium conditioned by the transfected cells. Preliminary data indicate the increased accumulation is not due to a change in half-life but to an increased synthetic and/or secretion rate.

DISCUSSION

We have found a region within the factor VIII molecule, the B domain as described by Vehar *et al.* (5), that exhibits an unusually high degree of porcine–human amino acid sequence divergence. In addition, up to 880 amino acids from this region, almost 38% of the protein, can be removed without a concomitant loss of procoagulant activity as measured *in vitro*. This is highly reminiscent of factor V, another blood clotting factor that is structurally related and acts analogously to factor VIII (15). This plasma glycoprotein is synthesized as a single-chain precursor of 330 kDa and is proteolytically activated by thrombin or factor Xa to yield factor Va (16, 17). Significant levels of factor Va activity are obtained when the NH_2-terminal (94 kDa) and COOH-terminal (74 kDa) thrombin cleavage fragments are reconstituted in the presence of divalent cations (18).

What role can be ascribed to the B domain? As an activation peptide it is an unusually large fraction of the molecule. The activation peptides for the serine proteases of the blood clotting cascade are generally much smaller. Specifically, the activation peptide of human factor X is 52 residues of a 448-amino acid zymogen. Interestingly, the bovine–human amino acid sequence homology is a mere 14% within the activation peptide, whereas much higher overall (19).

It is possible that the B domain of factor VIII is important for interaction with the von Willebrand factor (vWF), a large plasma glycoprotein that noncovalently associates with factor VIII in plasma. This interaction is important, since patients lacking von Willebrand factor also have severely reduced levels of plasma factor VIII. It will be interesting to determine whether the abridged forms of factor VIII bind to vWF. Other interesting possibilities for the function of this region are that it may be involved in intracellular processing or storage in the cells that normally process factor VIII *in vivo*, or that the B domain (or proteolytic products derived therefrom) has procoagulant, anticoagulant, or vasoactive properties heretofore unknown. For example, the vasodilator bradykinin is a nonapeptide proteolytically released from high molecular weight kininogen, which, like factor VIII, participates in the intrinsic pathway of blood coagulation.

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Biochemistry

Characterization of a genetically engineered inactivation-resistant coagulation factor VIIIa

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ABSTRACT **Individuals with hemophilia A require frequent infusion of preparations of coagulation factor VIII. The activity of factor VIII (FVIII) as a cofactor for factor IXa in the coagulation cascade is limited by its instability after activation by thrombin. Activation of FVIII occurs through proteolytic cleavage and generates an unstable FVIII heterotrimer that is subject to rapid dissociation of its subunits. In addition, further proteolytic cleavage by thrombin, factor Xa, factor IXa, and activated protein C can lead to inactivation. We have engineered and characterized a FVIII protein, IR8, that has enhanced *in vitro* stability of FVIII activity due to resistance to subunit dissociation and proteolytic inactivation. FVIII was genetically engineered by deletion of residues 794-1689 so that the A2 domain is covalently attached to the light chain. Missense mutations at thrombin and activated protein C inactivation cleavage sites provided resistance to proteolysis, resulting in a single-chain protein that has maximal activity after a single cleavage after arginine-372. The specific activity of partially purified protein produced in transfected COS-1 monkey cells was 5-fold higher than wild-type (WT) FVIII. Whereas WT FVIII was inactivated by thrombin after 10 min *in vitro*, IR8 still retained 38% of peak activity after 4 hr. Whereas binding of IR8 to von Willebrand factor (vWF) was reduced 10-fold compared with WT FVIII, in the presence of an anti-light chain antibody, ESH8, binding of IR8 to vWF increased 5-fold. These results demonstrate that residues 1690–2332 of FVIII are sufficient to support high-affinity vWF binding. Whereas ESH8 inhibited WT factor VIII activity, IR8 retained its activity in the presence of ESH8. We propose that resistance to A2 subunit dissociation abrogates inhibition by the ESH8 antibody. The stable FVIIIa described here provides the opportunity to study the activated form of this critical coagulation factor and demonstrates that proteins can be improved by rationale design through genetic engineering technology.**

Hemophilia A results from the quantitative or qualitative deficiency of coagulation factor VIII (FVIII), necessitating exogenous replacement by either plasma- or recombinant-derived FVIII preparations. FVIII has a domain organization of A1-A2-B-A3-C1-C2 and is synthesized as a 2,351-aa single-chain glycoprotein of 280 kDa from which a signal peptide is cleaved upon translocation into the lumen of the endoplasmic reticulum (1–3). Whereas the A and C domains exhibit 35–40% amino acid identity to each other and to the A and C domains of coagulation factor V, the B domain is not homologous to any known protein. Intracellular proteolytic processing within the B domain after residue Arg-1648 generates an 80-kDa light chain (domains A3-C1-C2) and a heterogeneous-sized heavy chain of 90–200 kDa (domains A1-A2-B). The heavy and light chains are associated as a heterodimer through a divalent metal-ion-dependent linkage between the A1 and A3 domains.

In plasma, FVIII circulates in an inactive form bound to von Willebrand factor (vWF) through noncovalent interactions and requires proteolytic cleavage by thrombin or factor Xa for activation (4–8). Upon proteolytic cleavage by thrombin, activated FVIII (FVIIIa) is released from vWF and functions to increase the V_{max} of factor IXa proteolytic activation of factor X by at least 4 orders of magnitude in the presence of phospholipid and calcium (9, 10). Thrombin cleavage after Arg residues 372, 740, and 1689 activates FVIII coagulant activity, and this coincides with generation of a FVIIIa heterotrimer consisting of the A1 subunit in a divalent-metal-ion-dependent association with the thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit associated with the A1 domain through an ionic interaction (Fig. 1*A*) (4, 11–13). This FVIIIa heterotrimer is unstable and subject to rapid inactivation through dissociation of the A2 subunit under physiological conditions (14).

Previous studies demonstrated that cleavages after Arg-372 and Arg-1689 are the most critical for efficient functional activation, whereas cleavage after Arg-740 was not required (13, 15–18). Cleavage after Arg-1689 removes an acidic amino acid-rich region from Arg-1648 to Arg-1689, and is necessary for dissociation of FVIIIa from vWF and makes FVIIIa available for interaction with phospholipids (4, 19–21). Cleavage after residue Arg-740 releases the heavily glycosylated B domain. Previous studies demonstrated that the B domain of FVIII is dispensable for FVIII cofactor activity (22–24). Genetically engineered FVIII molecules that have varying degrees of B domain deletion yield functional FVIII molecules that are more efficiently expressed in mammalian cells (22, 23, 25, 26). These deletion molecules exhibit typical thrombin activation that correlates with cleavage after Arg-372, Arg-740, and Arg-1689, generating a FVIIIa heterotrimer that is indistinguishable from wild-type FVIII (FVIII WT) and also is subject to rapid inactivation though dissociation of the A2 domain subunit.

With the greater understanding of the structural requirements for FVIII cleavage and activation, we have designed a functional B domain deletion FVIII that is not susceptible to rapid dissociation of the A2 domain subunit. We tested the hypothesis that fusion of the A2 subunit with the A3-C1-C2 light chain would yield a molecule in which only a single cleavage after Arg-372 would be necessary for functional activation. Under this hypothesis, the resultant FVIIIa molecule would not be susceptible to A2 subunit dissociation because the A2 subunit would be covalently attached to the light chain. We have generated such a molecule, inactivation-resistant factor VIII (IR8), which is resistant to spontaneous inactivation and exhibits a higher specific activity than FVIII WT. The results demonstrate the importance of A2 subunit dissociation in limiting the activity of FVIIIa.

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Abbreviations: FVIII, factor VIII; FVIIIa, thrombin-activated factor VIII; WT, wild type; vWF, von Willebrand factor; IR8, inactivation-resistant factor VIII; APTT, activated partial thromboplastin time.

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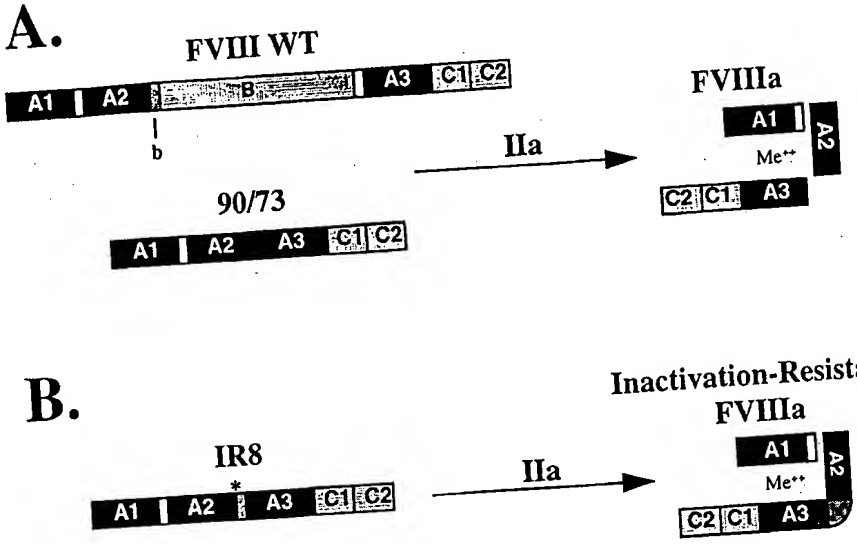

FIG. 1. Structural domains of FVIII WT, B domain-deleted and inactivation-resistant FVIII (IR8). (*A*) A schematic representation of FVIII WT and B domain-deleted FVIII (90/73) domain structure and their predicted FVIIIa heterotrimeric subunit structure after thrombin activation (IIa). (*B*) A similar representation of IR8 and its predicted heterodimeric subunit structure after thrombin activation. Me^{++} indicates a divalent metal ion necessary for heavy and light chain association. *, indicates the missense mutation at residue 740 predicting resistance to thrombin cleavage. b indicates the 54 amino acids of B domain retained in the IR8 construct. White boxes represent the acidic regions at the A1-A2 and the B-A3 junctions.

MATERIALS AND METHODS

Materials. Anti-heavy chain factor VIII mAb (F-8), F-8 conjugated to CL-4B Sepharose, and purified recombinant factor VIII protein were a gift from Debra Pittman (Genetics Institute, Cambridge, MA). Anti-human vWF horseradish peroxidase-conjugated rabbit antibody was purchased from Dako. Anti-light chain factor VIII mAbs, ESH-4 and ESH-8, were purchased from American Diagnostica (Greenwich, CT). Factor VIII-deficient and normal pooled human plasma were obtained from George King Biomedical (Overland Park, KS). Activated partial thromboplastin (Automated APTT reagent) and $CaCl_2$ were purchased from General Diagnostics Organon Teknika (Durham, NC). Human thrombin and aprotinin were purchased from Boehringer Mannheim. [^{35}S]methionine (>1,000 Ci/mmol) was purchased from Amersham. En^{3}Hance was purchased from DuPont. Fetal bovine serum was purchased from PAA Laboratories (Newport Beach, CA). DMEM, methionine-free DMEM, OptiMEM, biotin *N*-hydroxy succinimide ester, and streptavidin-horseradish peroxidase conjugate were purchased from GIBCO/BRL. *O*-phenylendiamine dihydrochloride was purchased from Sigma.

Plasmid Mutagenesis. Mutagenesis was performed within the mammalian expression vector pMT_2 (27) containing the FVIII cDNA (pMT_2VIII). Mutant plasmids were generated through oligonucleotide site-directed mutagenesis by using the PCR as described (28). The plasmid designated 90/80 is FVIII WT cDNA sequence in which the B domain was deleted (del741–1648) and was described previously (29). The plasmid designated 90/73 is FVIII WT cDNA sequence in which the B domain and the acidic region of the light chain have been deleted (del741–1689) and was described previously (20).

Construction 1 (90/73 R740K). Vector $pMT_2$90/73 was used as the DNA template. Oligonucleotide-directed mutagenesis was used to create a *Kpn*I–*Apa*I PCR fragment in which codon 740 was mutated from AGA to AAA, predicting an amino acid substitution of lysine for arginine, and was ligated into *Kpn*I–*Apa*I-digested $pMT_2$90/73.

Construction 2 (90/b/73 R740K). Vector pMT_2VIII was used as the DNA template. Oligonucleotide-directed mutagenesis was used to create a *Kpn*I/b/*Mlu*I PCR fragment (where b represents DNA sequence encoding for amino acid residues 741–793 of the WT sequence followed by an *Mlu*I site predicting amino acids threonine and arginine at residues 794 and 795/1689), which was ligated into the *Kpn*I–*Mlu*I-digested vector pMT_2VIII/1689*Mlu*I. Vector pMT_2VIII/1689*Mlu*I was generated by the heteroduplex procedure to introduce an *Mlu*I site at residue 1689 to facilitate cloning as described previously (30).

Construction 3 (90/b/73 R740A). Vector 90/b/73 R740K was used as the DNA template. Oligonucleotide-directed mutagenesis was used to create a *Kpn*I/b/*Apa*I fragment in which codon 740 was mutated from AAA to GCA, predicting an amino acid substitution of alanine for lysine, which was ligated into *Kpn*I–*Apa*I-digested $pMT_2$90/73.

Construction 4 (90/b/73 R336I/R740A). Vector PMT_2VIII/R336I was digested with *Spe*I and *Kpn*I. The fragment was ligated into *Spe*I–*Kpn*I-digested 90/b/73 R740A.

Construction of IR8 (90/b/73 R336I/R562K/R740A). Vector PMT_2VIII/R562K was digested with *Bgl*II and *Kpn*I. The fragment was ligated into *Bgl*II–*Kpn*I-digested 90/b/73 R336I/R740A. The plasmid containing the WT FVIII cDNA sequence was designated FVIII WT. All plasmids were purified by centrifugation through cesium chloride and characterized by restriction endonuclease digestion and DNA sequence analysis.

DNA Transfection and Analysis. Plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method as described (31). Conditioned medium was harvested at 64 hr posttransfection in the presence of 10% fetal bovine serum. FVIII activity was measured by one-stage APTT clotting assay on a Medical Laboratory Automation Electra 750 (Pleasantville, NY). Protein synthesis and secretion were analyzed by metabolically labeling cells at 64 hr posttransfection for 30 min with [^{35}S]methionine (300 μCi/ml in methionine-free medium), followed by a chase for 4 hr in medium containing 100-fold excess unlabeled methionine and 0.02% aprotinin. Cell extracts and conditioned medium were harvested, and immunoprecipitations were performed and analyzed as described (31).

Protein Purification. Partially purified IR8 protein was obtained from 200 ml of conditioned medium from transiently transfected COS-1 cells by immunoaffinity chromatography (32), yielding 300–1,500 ng per purification. FVIII WT protein was purified in parallel from stably transfected Chinese hamster ovary cells. The proteins eluted into the ethylene glycol-containing buffer were dialyzed and concentrated against a polyethylene glycol (M_r ≈15–20,000)-containing buffer (14) and stored at −70°C.

FVIII Activity and Antigen Assay. FVIII activity was measured in a one-stage APTT clotting assay by reconstitution of human FVIII-deficient plasma. For thrombin activation, protein samples were diluted into 50 mM Tris·HCl, pH 7.5/150 mM NaCl/2.5 mM $CaCl_2$/5% glycerol and incubated at room temperature with 1

unit/ml of thrombin. After incubation for increasing periods of time, aliquots were diluted and assayed for FVIII activity. One unit of FVIII activity is the amount measured in 1 ml of normal human pooled plasma. FVIII antigen was quantified using a sandwich ELISA method using anti-light chain antibodies ESH-4 and ESH-8 (33). Recombinant factor VIII protein purified in parallel was used as a standard.

FVIII-vWF Complex ELISA. Immulon 2 microtiter wells (Dynatech) were coated with F-8 antibody at a concentration of 2 μg/ml overnight at 4°C in a buffer of 0.05 M sodium carbonate/bicarbonate, pH 9.6. Wells were washed with TBST (50 mM Tris·HCl,pH 7.6/150 mM NaCl/0.05% Tween 20) then blocked with 3% BSA in TBST. Protein samples were diluted in TBST, 3% BSA, 1% factor VIII-deficient human plasma +/− ESH8 (molar ratio of ESH8/FVIII protein = 2:1). Samples were incubated for 2 hr at 37°C in 1.7-ml microfuge tubes and then incubated for an additional 2 hr in the blocked and washed microtiter wells. FVIII/vWF complexes were detected as described (34).

RESULTS

Generation of Inactivation Resistant FVIII. Variable deletions between residues 740 and 1648 within FVIII yield molecules with WT activity that generate a heterotrimer after cleavage by thrombin (26, 30, 35). Further deletion of the acidic region in the light chain (740–1689, termed 90/73) also yielded a functional molecule that generated a heterotrimer after thrombin cleavage; however, this molecule had a significantly reduced affinity for vWF (20). We tested the requirement for cleavage at the 740/1689 junction within the 90/73 deletion molecule by site-directed mutagenesis to change the Arg-740/1689 to Lys. Thrombin treatment of the resultant secreted molecule generated a heterodimer due to a single cleavage after Arg-372. This molecule would be resistant to dissociation of the A2 subunit, because it was fused to the light chain. However, the resultant molecule was not active in the one-stage APTT clotting assay either before or after thrombin cleavage. We hypothesized that a conformational constraint of the A2 domain may prevent activity of the 90/73 molecule having the A2 domain juxtaposed to the A3 domain. We therefore introduced a "spacer" of B domain (b) consisting of the amino acid residues 741–793 of the WT sequence followed by an *Mlu*I site (for cloning purposes), predicting amino acids Thr and Arg at residues 794 and 795/1689. This construct 90/b/73 is similar to a previously characterized B domain deletion FVIII (FVIII-LA) that is efficiently secreted and susceptible to thrombin cleavage and activation (26). The A2-b junction then was mutated from Arg to Lys or Ala at residue 740 to prevent thrombin cleavage at this site (Fig. 1*B*). Thus, upon incubation with thrombin, cleavage should occur only after Arg-372 to generate a FVIIIa heterodimer.

Both thrombin and factor Xa can cleave after Arg-336 and inactivate FVIII by loss of the acidic region at the carboxy terminus of the A1 domain. This acidic region may be important for interaction with the A2 subunit (36). To prevent cleavage and loss of the acidic region, Arg-336 was mutated to Ile because this amino acid change previously was shown to abrogate cleavage (14). Finally, activated protein C can inactivate FVIIIa by proteolytic cleavage after Arg-336 and Arg-562 (37). Cleavage at either of these sites by activated protein C would generate a heterotrimer of subunits that would be subject to inactivation through A2-subunit dissociation. Thus, Arg-562 was mutated to Lys to abrogate cleavage at this site. Previous observations with a full-length FVIII construct containing Arg-336-Ile and Arg-562-Lys demonstrated that these missense mutations did not interfere with synthesis, secretion, or functional activation and were resistant to APC-mediated cleavage at these sites (K. Amano and R.J.K., unpublished data). The final construct FVIII (del795–1689/Arg-336-Ile/Arg-562-Lys/Arg-740-Ala) was termed IR8.

Synthesis and Secretion of IR8. The synthesis and secretion of FVIII WT and the various inactivation-resistant mutants were compared by transient DNA transfection into COS-1 monkey cells. At 64 hr posttransfection, the rates of synthesis were analyzed by immunoprecipitation of cell extracts from [^{35}S]methionine pulse-labeled cells. Intracellular FVIII WT was detected in its single-chain form and migrated at approximately 250 kDa (Fig. 2*A*, lane 1). The B domain deletion mutant 90/80 (del741–1648) migrated at ≈170 kDa (Fig. 2*A*, lane 3). 90/73 migrated slightly faster due to the additional deletion of the residues in the acidic region of the light chain (Fig. 2*A*, lane 5). All of the 90/b/73-based constructs, including IR8, (Fig. 2*A*, lanes 7, 9, and 11) exhibited similar band intensity to the 90/80 and 90/73 constructs, suggesting that the multiple missense mutations did not interfere with efficient protein synthesis. After a 4-hr chase period, the majority of FVIII WT was lost from the cell extract (Fig. 2*A*, lane 2) and was recovered from chase-conditioned medium as a 280-kDa single chain, a 200-kDa heavy chain, and an 80-kDa light chain (Fig. 2*B*, lane 3). All of the inactivation-resistant mutants were recovered from the chase-conditioned medium as single-chain species (Fig. 2*B*, lanes 5, 7, 9, and 11). Therefore the various alterations of the FVIII construct did not significantly affect secretion.

Structural Stability of IR8 After Thrombin Cleavage. The labeled FVIII proteins immunoprecipated from the chase-conditioned medium were incubated with thrombin (1 unit/ml) for 30 min before SDS/PAGE analysis. FVIII WT was efficiently cleaved into a heterotrimer of fragments consisting of a 50-kDa A1 subunit, 43-kDa A2 subunit, and 73-kDa thrombin-cleaved light chain, A3-C1-C2 (Fig. 2*B*, lane 4). 90/73 WT also was cleaved into a heterotrimer of subunits similar to FVIII WT (Fig. 2*B*, lane 6). 90/73 Arg-740-Lys generated a heterodimer of thrombin-cleaved subunits consistent with a 50-kDa A1 subunit and an A2-A3-C1-C2 fused light chain (Fig. 2*B*, lane 8). 90/b/73 Arg-740-Lys generated thrombin cleavage fragments consistent with two heteromeric species, a 50-kDa A1 and a 120-kDa A2-b-A3-C1-C2 heterodimer, as well as a 43-kDa A2 subunit and an ≈85-kDa fragment consistent with a b-A3-C1-C2 fused light chain (Fig. 2*B*, lane 10). The appearance of the A2 subunit after incubation with thrombin suggested that the conservative mutation Lys-740 did not completely abrogate thrombin cleavage in the presence of the b spacer. With the nonconserved missense mutation to Arg-740-Ala a stable heterodimeric species was demonstrated (Fig. 2*B*, lane 12). This stable heterodimeric structure after thrombin cleavage was maintained for IR8 with subsequent additions of the missense mutations Arg-336-Ile and Arg-562-Lys.

Functional Stability of IR8 After Thrombin Activation Correlates with Increased Specific Activity. Having demonstrated the structural integrity of the IR8 heterodimer upon thrombin cleavage, the functional consequence of this modification on activation and inactivation was examined in an *in vitro* functional assay. Immunoaffinity-purified FVIII WT and IR8 were incubated with thrombin and assayed for FVIII activity by a one-stage APTT clotting assay. Upon treatment with thrombin, FVIII WT was maximally activated within 10 sec and then rapidly inactivated over the next 5 min. IR8 reached peak activity after 30-sec incubation with thrombin (Fig. 3), suggesting a modestly reduced sensitivity to thrombin activation compared with FVIII WT. In addition, the peak activity for thrombin-activated IR8 was lower (75 ± 7% of peak activity obtained from thrombin-activated FVIII WT, $n = 3$), suggesting some reduced efficiency as a cofactor. However, IR8 demonstrated significant stabilization of peak activity over the first 10-min incubation with thrombin (67 ± 5% of peak IR8 activity, $n = 3$), where activity of FVIII WT was less than 5%. Although a gradual loss of peak IR8 activity was seen with prolonged incubation with thrombin, IR8 still retained ≈38% of peak activity after 4-hr incubation with thrombin.

Immunoaffinity-purified FVIII WT and IR8 were assayed for FVIII activity using a standard one-stage APTT clotting assay. Antigen determinations were made using a FVIII light

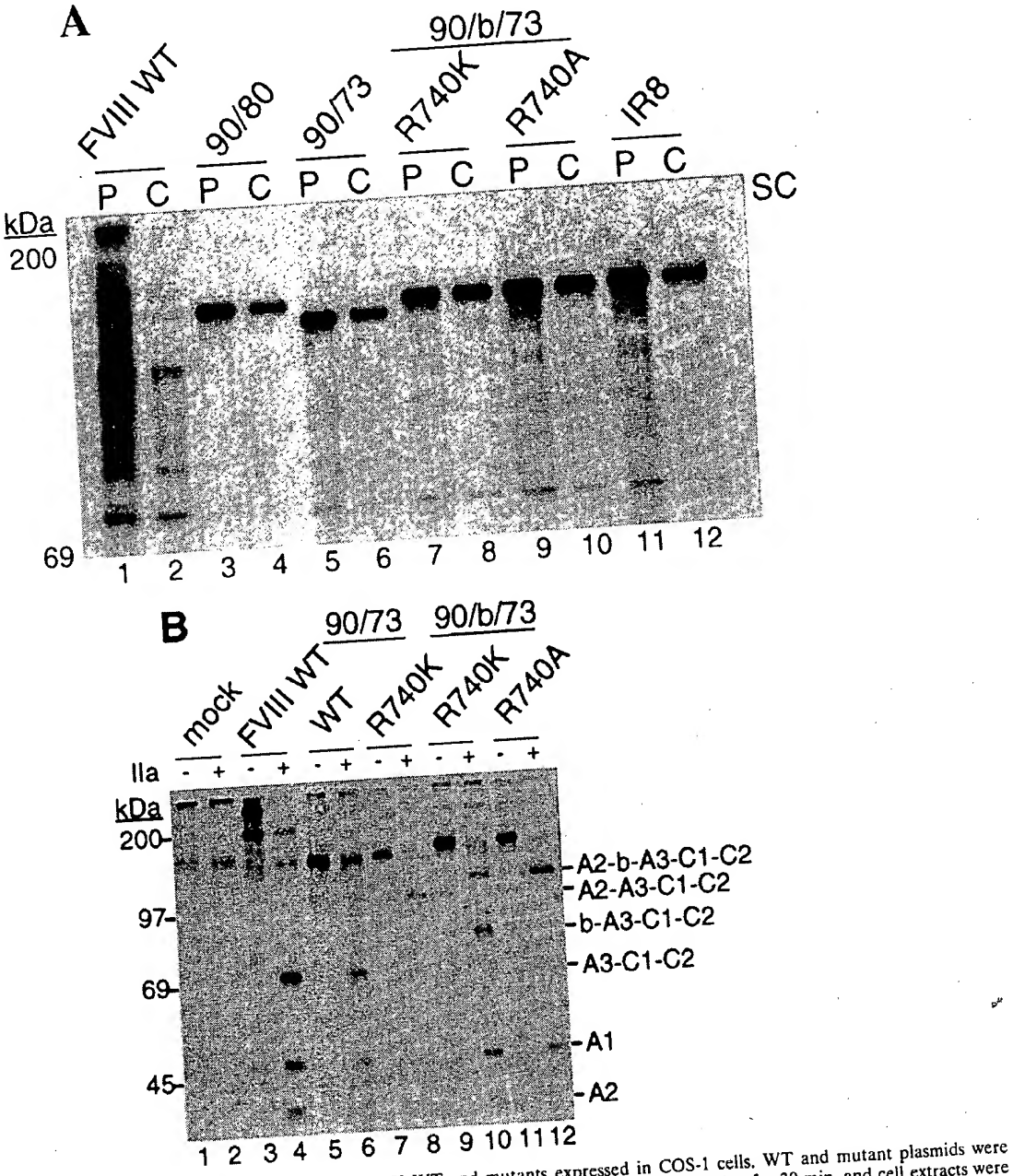

FIG. 2. Synthesis, secretion, and thrombin cleavage of FVIII WT and mutants expressed in COS-1 cells. WT and mutant plasmids were transfected into COS-1 monkey cells. At 64 hr posttransfection, cells were pulse-labeled with [^{35}S]methionine for 30 min, and cell extracts were harvested. Duplicate labeled cells were chased for 4 hr in medium containing excess unlabeled methionine, and then cell extracts and conditioned medium were harvested. Equal proportionate volumes of cell extract and conditioned medium were immunoprecipated with anti-FVIII-specific antibody, and equal aliquots were analyzed by SDS/PAGE. A duplicate sample of immunoprecipitated labeled protein was incubated with thrombin (1 unit/ml) for 30 min at 37°C before SDS/PAGE analysis. Mock indicates cells that did not receive plasmid DNA. Cell extract pulse (P) and chase (C) (*A*). Chase conditioned media before (*B*, −) and after thrombin digestion (*B*, +). The migration of FVIII WT from the cell extracts is indicated at the right as a single chain (SC). FVIII from the conditioned medium is indicated at the far right as single chain (SC), heavy chain (HC), and light chain (LC) forms. The migration of the mutants from the cell extracts and conditioned medium is indicated on the right of each image by their predicted domain structure. Molecular mass markers are shown on the left of each image.

chain-based ELISA. The specific activity values for IR8 then were calculated based on a correction for its molecular weight. IR8 was observed to have a 5-fold increased specific activity compared with FVIII WT (102 ± 43 vs. 18.6 ± 7.4 units/mg).

IR8 Is Not Inhibited by the mAb ESH8. Characterization of a FVIII inhibitory mAb ESH8 demonstrated that its inhibition was dependent on binding of FVIII to vWF (38–40). Because IR8 had deleted residues required for high-affinity vWF binding, we tested the inhibitory effect of the ESH8 antibody on IR8. Because ESH8 inhibits FVIII only in the presence of vWF, IR8 first was examined for its affinity for vWF. Immunoaffinity-purified FVIII WT and IR8 proteins were assayed for their binding to vWF in solution. FVIII WT demonstrated saturable vWF binding as FVIII concentrations increased to 50

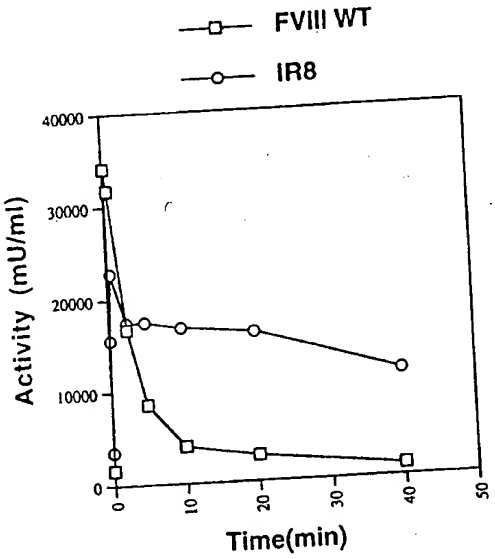

FIG. 3. Activation and inactivation of FVIII and IR8 by thrombin. Partially purified FVIII WT and IR8 proteins (1 nM) were incubated with thrombin (1 unit/ml) at room temperature and assayed over time for FVIII activity by APTT. The results are from a single thrombin activation experiment and are typical of multiple independent experiments.

ng/ml (Fig. 4). IR8 demonstrated approximately 10-fold lower affinity for vWF, consistent with the deletion of the light chain acidic region. However, in the presence of ESH8, IR8 demonstrated significantly increased affinity for vWF.

The functional impact of this ESH8-induced binding of IR8 to vWF complex was evaluated by assaying for FVIII activity using the one-stage clotting assay (Table 1). In the absence of ESH8, immunoaffinity-purified FVIII WT and IR8 demonstrated minimal loss of activity over a 4-hr incubation at 37°C with FVIII-deficient plasma. In the presence of ESH8, FVIII WT activity was inhibited to 30%, whereas IR8 retained 100% of its initial activity. These results suggest that inactivation of WT FVIII in the presence of ESH8 may be due to A2 subunit dissociation, and IR8 is resistant to inactivation by ESH8 because it is not susceptible to A2 subunit dissociation.

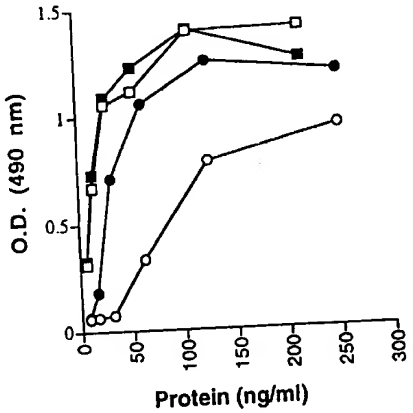

FIG. 4. FVIII-vWF binding of partially purified FVIII proteins as determined by ELISA. FVIII WT and IR8 proteins were purified through immunoaffinity chromotography and analyzed for binding to vWF after incubation with human FVIII-deficient plasma. O.D. represents the absorbance obtained through the detection of FVIII-vWF complexes by an anti-vWF-horseradish peroxidase conjugate antibody in the presence of *O*-phenylendiamine dihydrochloride. Squares, FVIII WT; circles, IR8; open symbols, absence of ESH8; closed symbols, presence of ESH8.

DISCUSSION

With an increased understanding of the requirements for FVIII function, studies have attempted to produce improved FVIII molecules for replacement therapy for patients with hemophilia A. Strategies investigated thus far have included the deletion or modification of FVIII sequences, resulting in more efficient expression. These strategies, although offering potential for more efficient manufacturing of recombinant protein or more efficient delivery through gene therapy, have not increased FVIII plasma half-life or specific activity; either of which could potentially reduce the amount of FVIII required for efficient replacement for hemorrhagic events. Decreasing the amount of FVIII protein for replacement therapy would have tremendous benefit to individuals with hemophilia A because this would not only reduce the cost of treatment but should minimize inhibitor antibody development that occurs in response to the heavy antigen load of FVIII being recognized as a foreign protein. Our studies have focused on the potential to develop an improved FVIII therapeutic by increasing the specific activity of recombinant FVIII.

Previous studies on the requirements for functional activity of FVIII demonstrated that cleavage after Arg residues 372 and 1689 both were required for activation of factor VIII and that the B domain was not required for functional activity (13, 22–24). Deletion of residues 741-1689 yielded a functional molecule (90/73) that displayed WT thrombin cleavage and activation, resulting in a heterotrimer similar to WT FVIIIa that was susceptible to rapid dissociation of the A2 subunit. We tested the hypothesis whether mutagenesis of the 740-1690 cleavage site within 90/73 would yield a functional molecule that was activated by thrombin as a result of a single cleavage at Arg-372. However, mutation of the 740-1690 junction within 90/73 destroyed its activity, although the resultant molecule was susceptible to thrombin cleavage at Arg-372. These results suggested that either cleavage at the amino terminus of the light chain is required to elicit procoagulant activity or that the A2 subunit requires an appropriate distance from the A3 domain in the light chain. When a 54-aa spacer, comprised of B domain sequence, was introduced between the A2 domain and the light chain, near WT activity, as well as thrombin activation, was restored. These results demonstrate both that thrombin activation of FVIII does not require cleavage at the amino terminus of the light chain and that there is a spatial requirement between the light chain and the A2 domain for FVIII activity. The spatial requirement may reflect the two factor IXa interaction sites identified at residues Ser-558–Glu-565 in the A2 domain that interacts with the factor IXa serine protease domain (41, 42) and Glu-1811–Lys-1818 in the A3 domain that interacts with the factor IXa epidermal growth factor 1 domain (43, 44). Most significantly, the resultant molecule IR8 was cleaved at a single site after Arg-372 to generate procoagulant activity and was resistant to inactivation mediated by A2 domain dissociation. These results support the importance of A2 subunit dissociation as a mechanism for inactivation of FVIIIa, as originally proposed by Lollar and Parker (45). The importance of cleavage at residue 372 previously was demonstrated by identification of mutants in Arg-372 that result in hemophilia A (16) and by analysis of site-directed mutants of Arg-372 (13). Interestingly there was a ≈25% loss of IR8 activity within the first 30 sec to 2.5 min after thrombin activation followed by only a very gradual loss of activity occurring over hours. We propose that noncleavage-mediated inactivation is responsible for the immediate loss of peak activity. It is possible that the "spacer" length or composition used in this form of IR8 is not optimal. This

Table 1. ESH8 does not inhibit IR8 activity in presence of vWF

Protein	% of initial activity	
	−ESH8	+ESH8
FVIII WT	92 ± 3	29 ± 13
IR8	101 ± 2	120 ± 27

possibility also is supported by the observation that the peak activity observed immediately after thrombin activation is less than that obtained with FVIII WT. Perhaps some unfolding of the IR8 protein occurs after activation and precludes efficient interaction with factor IXa. Further analysis of additional molecules based on IR8 having alternate spacer lengths or amino acid content may optimize the inactivation resistance.

Initial studies supported that vWF interacts with the acidic amino acid-rich amino terminus of the FVIII light chain. mAbs to residues 1670–1689 inhibit interaction with vWF (46–48) and deletion of a portion of this region destroys high-affinity vWF interaction (47). However, the isolated acidic peptide 1648–1689 was incapable of binding vWF, suggesting the vWF interaction requires other regions of the FVIII molecule. We have shown that deletion of the acidic amino acid-rich region in the light chain in IR8 yields a molecule that has 10-fold reduced interaction with vWF. More recently, it was demonstrated that antibodies to the carboxy-terminus of the C2 domain of FVIII inhibit vWF binding and support that the C2 domain is also important for vWF interaction. One C2 domain antibody, ESH8, reacts with a C2 domain epitope between residues 2248–2285 and is inhibitory only in the presence of vWF. We have shown that IR8 binding to vWF is increased approximately 5-fold in the presence of the ESH8 antibody. These results support that the high-affinity vWF binding site in IR8 is intact but its conformation is inadequate to support high-affinity vWF interaction. We propose that ESH8 antibody binding to IR8 induces a conformation that is capable of high-affinity vWF interaction. The ESH8-induced conformational change may be similar to the conformational change induced by the presence of the acidic amino acid-rich region in the FVIII light chain.

Frequently, hemophilia A patients treated with FVIII develop alloantibodies that react with the C2 domain and inhibit FVIII activity. ESH8 is one anti-C2 domain inhibitory antibody that does not interfere with binding of FVIII to vWF and for which the mechanism of FVIII inactivation has been studied. Studies support that ESH8 stabilizes the FVIII-vWF interaction to delay release of FVIIIa from vWF by 4-fold, so that A2 domain dissociation occurs before FVIIIa release from vWF (40). The unique stability of IR8 after thrombin-activation provided a useful reagent to test this hypothesis that previously was derived from kinetic measurements. We characterized the inhibitory effect of ESH8 on IR8 and found that IR8 was resistant to inactivation mediated by the ESH8 antibody. These results suggest that molecules similar to IR8 may provide therapeutic efficacy in patients that have FVIII inhibitor antibodies similar to ESH8.

We have tested the hypothesis that a heterodimeric FVIIIa molecule that is resistant to spontaneous inactivation through dissociation of the A2 subunit would increase its specific activity. The results show that IR8 exhibits a dramatically increased stability after thrombin activation under physiological conditions. The *in vitro* specific clotting activity of IR8 was increased approximately 5-fold over WT recombinant FVIII. These results support the importance for A2 domain dissociation in limiting FVIII activity *in vitro*. Future studies are required to evaluate the importance of A2 domain dissociation in limiting FVIII activity *in vivo*. These studies now can be performed with the IR8 molecule. Analysis of IR8 molecules that either contain or lack mutations at the APC inactivation sites at Arg-336 and Arg-562 will provide important information on the mechanism of inactivation of FVIII *in vivo*. Finally, the ability to isolate a stable form of FVIIIa will provide a critical reagent for structural studies as well as a means to study the importance of FVIIIa generation in thrombosis by infusion of stable FVIIIa into *in vivo* models of thrombotic disease.

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Construction and Characterization of an Active Factor VIII Variant Lacking the Central One-Third of the Molecule

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ABSTRACT: The primary structure of factor VIII consists of 2332 amino acids that exhibit 3 distinct structural domains, including a triplicated region (A domains), a unique region of 909 amino acids (B domain), and a carboxy-terminal duplicated region (C domains), that are arranged in the order A1-A2-B-A3-C1-C2. The B domain (residues 741–1648) of factor VIII is lost when factor VIII is activated by thrombin, which proteolytically processes factor VIII to active subunits of M_r 50 000 (domain A1), 43 000 (domain A2), and 73 000 (domains A3-C1-C2). To determine if the B domain is required for factor VIII coagulant activity, a variant was constructed by using recombinant DNA techniques in which residues 797–1562 were eliminated. This shortened the B domain from 909 to 142 amino acids. This variant factor $VIII_{des\text{-}797\text{-}1652}$ was expressed in mammalian cells and was found to be functional. The factor $VIII_{des\text{-}797\text{-}1562}$ protein was purified and shown to be processed by thrombin in the same manner as full-length factor VIII. The factor $VIII_{des\text{-}797\text{-}1562}$ variant also bound to von Willebrand factor (vWF) immobilized on Sepharose. These results indicate that most of the highly glycosylated B domain of factor VIII is not required for the expression of factor VIII coagulant activity and its interaction with vWF.

Factor VIII functions in the middle of the coagulation cascade as a cofactor for the activation of factor X by factor IXa (Jackson & Nemerson, 1980). Complete cDNA clones for human factor VIII have now been obtained and, along with protein characterization studies, have elucidated the structure of factor VIII (Wood et al., 1984; Toole et al., 1984; Fass et al., 1982; Fulcher & Zimmerman, 1982; Vehar & Davie, 1980; Rotblat et al., 1985; Eaton et al., 1986a). The factor VIII gene codes for a single-chain protein (M_r ~300 000) consisting of 2332 amino acids including 25 potential asparagine-linked glycosylation sites (Vehar et al., 1984). Analysis of the factor VIII sequence revealed 3 distinct domains, including a triplicated domain of ~330 amino acids (A domains), a unique region of 909 amino acids containing 19 asparagine-linked glycosylation sites (the B domain), and a carboxy-terminal duplicated domain of ~150 amino acids, which are arranged in the order A1-A2-B-A3-C1-C2 (Figure 1; Vehar et al., 1984). The single-chain form of factor VIII is readily proteolyzed in vitro and in vivo to multiple polypeptides having molecular weights ranging from 80 000 to 210 000 (Fulcher & Zimmerman, 1982; Rotblat et al., 1985; Weinstein et al., 1983; Eaton et al., 1986a; Figure 1). Amino-terminal sequence analysis shows that the M_r 210 000 and 80 000 proteins represent the amino- and carboxy-terminal regions of factor VIII, respectively (Vehar et al., 1984; Eaton et al., 1986a; Figure 1). Proteolysis within the carboxy-terminal region of the M_r 210 00 protein yields a series of proteins of M_r 90 000–180 000 (Toole et al., 1984; Vehar et al., 1984; Eaton et al., 1986a,b). The M_r 80 000 protein appears to form a metal (perhaps Ca^{2+}) linked complex with each of the M_r 90 000–210 000 proteins. Neither the M_r 80 000 protein nor the M_r 90 000–210 000 proteins have coagulant activity when separated (Eaton et al., 1986b).

The single-chain form and also the above-mentioned multiple-polypeptide form of factor VIII are proteolytically processed by thrombin to an active form (Fass et al., 1982; Fulcher et al., 1983; Eaton et al., 1986a) consisting of subunits with M_r 50 000 (domain A1), 43 000 (domain A2), and 73 000 (domains A3-C1-C2) (Fass et al., 1982; Eaton et al., 1986a; Figure 1). Between these functional regions of factor VIII is the highly glycosylated B domain (residues 742–1649) that appears to be proteolytically removed when factor VIII is activated. This suggests that this domain may not be required for factor VIII coagulant activity. To test this hypothesis, we

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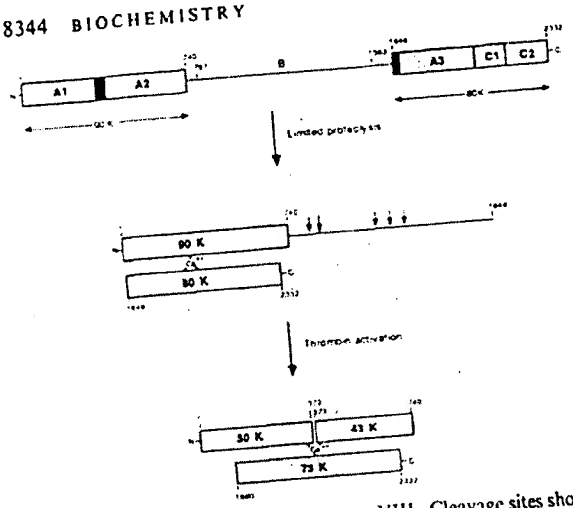

FIGURE 1: Proteolytic processing of factor VIII. Cleavage sites shown are taken from Eaton et al. (1986a) and Toole et al. (1984). Residues 797–1562 are removed in factor $VIII_{des\text{-}797\text{-}1562}$.

used recombinant DNA techniques to construct a factor VIII variant (factor $VIII_{des\text{-}797\text{-}1562}$ in which most of the B domain is removed. When this variant is expressed in mammalian cells, factor VIII activity is found in the culture medium, demonstrating that the highly glycosylated B domain is not needed for activity. Purification and characterization of the variant show that it is functionally similar to full-length factor VIII.

Materials and Methods

Factor VIII deficient and normal human plasmas were from George King Biomedical; platelin was from General Diagnostics; factor VIII chromogenic Coatest assay was from Helena; human α-thrombin was from Sigma Chemical Co. The plasmid pUC8 containing the CMV enhancer, promoter, and splice donor was obtained from Dr. B. Fleckenstein (Nurnberg, West Germany). DMEM and Hams F12 media were obtained from Flow Laboratories; Geneticin was from Gibco; Affigel-10 was from Bio-Rad.

$Factor\ VIII_{des\text{-}797\text{-}1562}$ Fusion Expression Plasmid. A deletion of the B domain of factor VIII cDNA (Wood et al., 1984) sequence was made by fusing the *Tth*111 I site at amino acid 796 to the *Bam*HI site at amino acid 1563 after filling both sites with DNA polymerase I. The expected junction was confirmed by DNA sequence analysis. This fusion construction deletes amino acids 797–1562 of the B domain. This factor VIII variant cDNA was expressed in a pML (Lusky & Botchan, 1981) vector containing transcriptional control region of the human cytomegalovirus (CMV). This 5′ control region includes the CMV enhancer, promoter, and splice donor sequence (Boshart et al., 1985; Thomsen et al., 1984; Sternberg et al., 1984). To complete the intron and provide a splice acceptor site, we synthesized a 99 base pair (bp) oligomer containing the sequence of the splice acceptor of the Ig variable region (Bothwell et al., 1981). The variant cDNA is followed by the poly(A) addition site and the transcription terminator of the early region of SV40 (Fiers et al., 1978). This plasmid is designated pF8CIS9080.

Transfection and Cell Culture. Mammalian kidney cells were cotransfected with pF8CIS9080 and pRSVneo (Gorman et al., 1983) by the calcium phosphate precipitation method (Graham & van der Eb, 1973). Two 60-mm dishes of cells were transfected with 1 μg of pF8CIS9080, 0.5 μg of pRSVneo, and 3.5 μg of salmon sperm DNA each. Forty eight hours later, cells were either assayed for transient expression

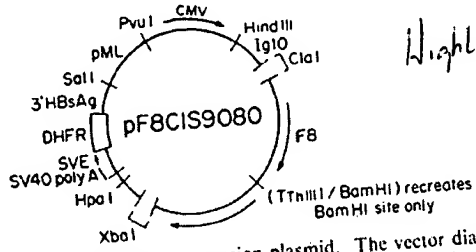

FIGURE 2: pF8CIS9080 expression plasmid. The vector diagram includes the Amp^R gene and procaryotic origin of replication from pML; the immediate early gene enhancer, promoter, and splice donor from the human cytomegalovirus; and an immunoglobin splice acceptor and the sequence for factor $VIII_{des\text{-}797\text{-}1562}$ flanked by the SV40 poly(A) addition site. Also included in this plasmid is the SV40-dhfr transcription unit (Kaufman & Sharp, 1982; Simonsen & Levinson, 1983).

by immunoperoxidase staining using a factor VIII monoclonal antibody (Wood et al., 1984; Gorman et al., 1985) or subcultured into 400 μg/mL Geneticin. Clones were subsequently pooled to give a mass population of cells. Expression of factor VIII from this new cell line, 90/80 cells, was determined by immunostaining of cells and assay of culture medium for factor VIII coagulant activity. Cells were cultured in an F12/DMEM mix containing 7% fetal calf serum. To obtain serum-free medium containing the factor $VIII_{des\text{-}797\text{-}1562}$ variant, the cells were cultured in F12/DMEM medium without serum for 48 h.

Purification and Characterization of the Factor $VIII_{des\text{-}797\text{-}1562}$ Variant. The factor VIII variant was purified by using DEAE-Sepharose and a factor VIII monoclonal antibody column as described for recombinant full-length factor VIII (Eaton et al., 1986b). The antibody (C7F7) reacts with the M_r 80000 protein of factor VIII (Wood et al., 1984). Factor VIII activity was assayed by coagulation analysis and by the factor VIII Coatest assay (Wood et al., 1984). Protein determinations were done by the method of Bradford (1976). For amino-terminal sequencing, the factor $VIII_{des\text{-}797\text{-}1562}$ variant (0.2–0.5 mg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (6–12%) (Laemmli, 1970), and proteins were detected by staining with Coomassie blue. Proteins were then gel-eluted (Hunkapiller et al., 1983) and sequenced by using an Applied Biosystems vapor phase sequencer (Hewick et al., 1982).

Results

Expression of the Functional Factor $VIII_{des\text{-}797\text{-}1562}$ Variant. The eukaryotic expression vector used to express factor $VIII_{des\text{-}797\text{-}1562}$, as described under Materials and Methods, is shown in Figure 2. The protein coding region lacks amino acid residues 797–1562 of the factor VIII protein (Figure 1). This expression plasmid was used to transfect mammalian kidney cells, and factor VIII expression was monitored by immunoperoxidase staining (Gorman et al., 1985) and by coagulation analysis. As shown in Table I, serum-free medium obtained from 90/80 cells shortens the coagulation time of hemophilic plasma, while medium from the parent cell line did not. This activity was inhibited by a polyclonal antibody raised against purified plasma-derived factor VIII. Factor VIII was also detected by immunostaining of 90/80 cells using a factor VIII monoclonal antibody (C7F7) Wood et al., 1984) (data not shown).

Characterization of the Factor $VIII_{des\text{-}797\text{-}1562}$ Variant. Analysis of purified factor $VIII_{des\text{-}797\text{-}1562}$ by SDS–PAGE shows that it consists primarily of two bands of M_r 80000 and 115000 (Figure 3). A small amount of the single-chain fusion having an M_r of ~200000 was also observed by SDS–PAGE.

Table I: Coagulant Activity of 90/80 Medium[a]

sample	clot time (s)
90/80 medium	58.9
90/80 medium preincubated with factor VIII polyclonal antibody	101.1
buffer	109.2
parent cell line medium	102.3

[a] 90/80 cells or the parent cell line was cultured in serum-free medium for 48 h, at which time it was harvested. The medium was diluted with 0.05 M Tris, pH 7.4, containing 0.01% BSA and assayed by coagulation analysis. For antibody neutralization, 90/80 medium (1 mL) was preincubated with 10 μg of a factor VIII polyclonal antibody for 45 min at 37 °C and subsequently assayed.

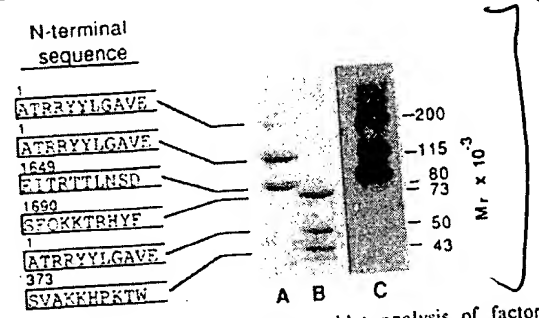

FIGURE 3: SDS-PAGE and Western blot analysis of factor $VIII_{des-797-1562}$. 15 μg of the variant (A) and 15 μg of the variant pretreated with 0.15 unit (50 ng) of thrombin (B) were resolved on a 6–12% SDS–polyacrylamide gel, and proteins were detected by staining with Coomassie blue. After SDS-PAGE, factor $VIII_{des-797-1562}$ was also transferred to nitrocellulose for Western blot analysis (Towbin et al., 1979) (C). A polyclonal antibody against plasma-derived factor VIII was used to detect proteins transferred to nitrocellulose. For amino-terminal sequencing, 0.5 mg of factor $VIII_{des-797-1562}$ or 0.5 mg of factor $VIII_{des-797-1562}$ incubated with 5 units (1.7 μg) of thrombin for 10 min at 37 °C and proteins were isolated and sequenced as described under Materials and Methods. The amino acid numbering shown is that for full-length factor VIII (Eaton et al., 1986a).

The M_r 200 000, 115 000, and 80 000 proteins were all detected by a factor VIII polyclonal antibody by Western blot analysis (Figure 3). Amino-terminal sequencing shows that the M_r 115 000 and 80 000 proteins represent the amino- and carboxy-terminal regions of the M_r 200 000 single-chain protein, respectively (Figures 1 and 3). The amino-terminal sequence obtained for the M_r 200 000 single-chain protein is identical with the DNA-predicted amino-terminal sequence of factor VIII (Wood et al., 1984). The sequence obtained from the M_r 80 000 protein is also identical with the amino-terminal sequence obtained from the M_r 80 000 protein of plasma-derived factor VIII (Figure 3; Eaton et al., 1986a).

The specific activity of purified factor $VIII_{des-797-1562}$ was found to be 4000–6000 units/mg. This is comparable to the specific activity of unactivated plasma-derived factor VIII (Fulcher & Zimmerman, 1982; Rotblat et al., 1985; Eaton et al., 1986a). Treatment of factor $VIII_{des-797-1562}$ with thrombin resulted in a 15–20-fold activation of coagulant activity (Figure 4). This activation was correlated with the cleavage of the M_r 200 000, 115 000, and 80 000 proteins to subunits with M_r 50 000, 43 000, and 73 000 (Figures 3 and 4). Initially, the M_r 200 000 protein appears to be cleaved to the M_r 115 000, 90 000, and 80 000 proteins. Proteolysis within the carboxy terminus of the M_r 115 000 protein removes the shortened B domain, generating the M_r 90 000 protein, which is subsequently cleaved at arginine-372 to generate the M_r 50 000 and 43 000 subunits (Figures 3 and 4). Concomitantly, proteolysis of the M_r 80 000 protein generates the M_r 73 000 subunit. Amino-terminal amino acid sequencing confirms that

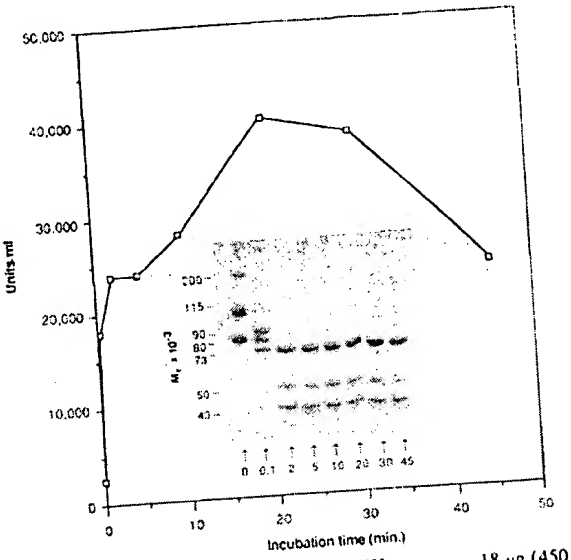

FIGURE 4: Thrombin activation of factor $VIII_{des-797-1562}$. 18 μg (450 μg/mL) of factor $VIII_{des-797-1562}$ in 0.05 M tris(hydroxymethyl)-aminomethane (Tris), pH 7.4, 0.15 M NaCl, 2.5 mM $CaCl_2$, and 5% glycerol was incubated with 60 ng (7.2 units/mL) of thrombin for 0.1–60 min at 37 °C. At the times indicated, an aliquot was removed and diluted (1/10 000)–(1/20 000) into 0.05 M Tris, pH 7.4, containing 0.01% bovine serum albumin (BSA) and assayed for coagulant activity. The remainder of the sample was brought to 0.5% SDS and heated at 90 °C for 5 min. Subsequently, the samples were resolved by SDS-PAGE (6–12%).

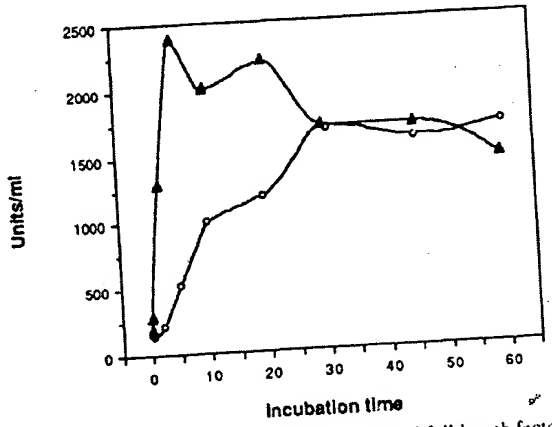

FIGURE 5: Comparison of factor $VIII_{des-797-1562}$ and full-length factor VIII activation. 2.4 μg (60 μg/mL) of either factor $VIII_{des-797-1562}$ (▲) or full-length factor VIII (○) was incubated with 4.8 ng (0.35 unit/mL) of thrombin for 0–60 min at 37 °C. At the times indicated, an aliquot was removed and diluted (1/1000)–(1/2000) into 0.05 M Tris, pH 7.4, containing 0.01% BSA and assayed for coagulant activity. Full-length factor VIII used in this experiment was purified from factor VIII concentrates as previously described (Eaton et al., 1986a).

the M_r 50 000, 43 000, and 73 000 subunits of the activated variant have the same amino-terminal sequence as their counterparts derived from plasma-derived factor VIII (Figure 3; Eaton et al., 1986a). This shows that factor $VIII_{des-797-1562}$ is processed by thrombin in a manner very similar to full-length factor VIII. By SDS-PAGE and staining with Coomassie blue, we could not detect the shortened B domain after thrombin activation. This may be the result of proteolysis to smaller peptides not resolved by the gel system used. Interestingly, we found that factor $VIII_{des-797-1562}$ was activated by thrombin at a significantly faster rate than full-length factor VIII. As shown in Figure 5, factor $VIII_{des-797-1562}$ was fully activated by thrombin (1/500 thrombin/factor VIII) in ~5

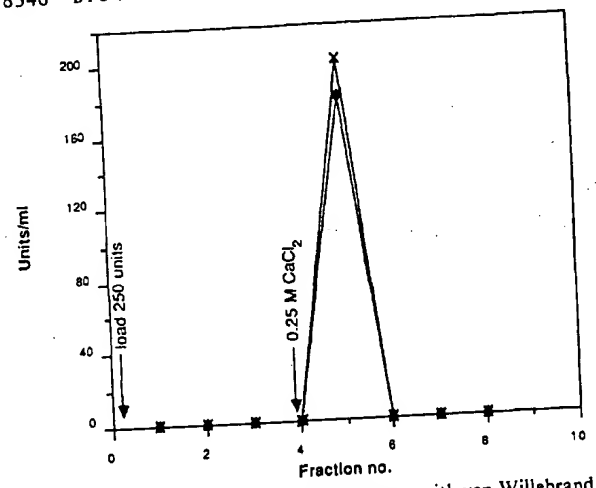

FIGURE 6: Interaction of factor $VIII_{des-797-1562}$ with von Willebrand factor. vWF was purified and coupled to Affigel-10 as previously described (Wood et al., 1984). The vWF column was equilibrated in 0.05 M Tris, pH 7.4, 0.15 M NaCl, and 2.5 mM $CaCl_2$. Factor $VIII_{des-797-1562}$ (×) or natural factor VIII (♦) (250 units of each) was passed through the column, which was subsequently washed with 3 column volumes of the above buffer. Factor $VIII_{des-797-1562}$ or natural factor VIII was eluted from the column with the above buffer containing 0.25 M $CaCl_2$, and 2-mL fractions were collected. Factor VIII coagulant activity was determined by coagulation analysis.

min while activation of full-length factor VIII by thrombin (1/500) took ~30 min.

In plasma, factor VIII circulates bound to von Willebrand factor (vWF) (Hoyer, 1981). In the absence of circulating vWF, such as the case in patients with vWF disease, factor VIII levels are depressed (Hoyer, 1981; Zimmerman et al., 1983). This is in part due to the lability and shortened half-life of factor VIII in the absence of circulating vWF (Brinkhous et al., 1985). When purified factor $VIII_{des-797-1562}$ was chromatographed on a vWF–Sepharose column, it bound to the resin (Figure 6). Subsequently, the variant was eluted with 0.25 M $CaCl_2$, which is known to dissociate vWF–Factor VIII complexes (Hoyer, 1981). The same result was obtained when full-length factor VIII was chromatographed on this column (Figure 6).

Discussion

On the basis of the observation that the B domain of factor VIII is proteolytically removed after activation of factor VIII by thrombin (Eaton et al., 1986a,b), we constructed a factor VIII variant in which 767 amino acids of the B domain were removed. This shortened the B domain from 909 amino acids containing 19 asparagine-linked glycosylation sites to 142 amino acids containing only 2 asparagine-linked glycosylation sites. This factor VIII variant was expressed and secreted from mammalian cells and found to be functional. While this paper was in preparation, the expression of an active factor VIII variant in which the B domain was shortened to 28 amino acids was reported (Toole et al., 1986). This factor VIII variant has not been purified or characterized as yet.

Factor $VIII_{des-797-1562}$ purified from 90/80 cell culture supernatants showed two major bands on SDS–PAGE of M_r 115000 and 80000 (Figure 3). These proteins probably form a metal-linked complex since ethylenediaminetetraacetic acid (EDTA) inactivated the variant (D. Eaton, unpublished observation). A small amount of the single-chain factor $VIII_{des-797-1562}$ of M_r 200000 was also observed by SDS–PAGE. The specific activity of factor $VIII_{des-797-1562}$ was found to be 4000–6000 units/mg, which is similar to the specific activity of full-length human factor VIII (Fulcher & Zimmerman, 1982; Rotblat et al., 1985; Eaton et al., 1986a). This is not unexpected since the specific activity of the isolated M_r 90000–80000 complex has the sample specific activity as the M_r 210000–80000 complex (D. Eaton, unpublished observations; Fay et al., 1986). This observation itself suggests that the presence of the highly glycosylated B domain has little effect on factor VIII coagulant activity.

Treatment of factor $VIII_{des-797-1562}$ with thrombin resulted in a 10–20-fold activation that was correlated with the generation of subunits with M_r 50000, 43000, and 73000 (Figure 4). This shows that factor $VIII_{des-767-1562}$ is activated and processed by thrombin in the same manner as full-length factor VIII. Factor $VIII_{des-797-1562}$ was also found to interact with vWF–Sepharose in a manner similar to that of full-length factor VIII. This indicates that the B domain may not mediate the binding of factor VIII to vWF, supporting recent findings that factor VIII binding to vWF is mediated by the carboxy-terminal region of factor VIII (Hamer et al., 1985).

Demonstration that factor $VIII_{des-797-1562}$ is active shows one more way in which factor VIII and factor V are functionally and structurally similar proteins (Church et al., 1984). Like factor VIII, factor V is initially synthesized as a large single-chain precursor of M_r 330000 that is proteolytically processed by thrombin to an active form (Mann et al., 1981). Activated factor V is a Ca^{2+}-linked complex consisting of two subunits with M_r 90000 (from the amino terminus) and 74000 (from the carboxy terminus) (Esmon, 1979). These subunits have been purified and are fully functional when reconstituted (Esmon, 1979). In the single-ohain form, these subunits are separated by a large highly glycosylated region that is proteolytically removed during activation (Mann et al., 1981; Esmon, 1979). Thus, the precursors of both factor V and factor VIII contain a large (~100 kilodaltons), highly glycosylated domain separating the functional domains of these proteins.

We find that the rate of thrombin activation of factor $VIII_{des-797-1562}$ was faster than that for full-length factor VIII, indicating that lower concentrations of thrombin are necessary to activate factor $VIII_{des-797-1562}$ when compared to full-length VIII (Figure 5). Whether this difference is enough to alter the functional properties of factor $VIII_{des-797-1562}$ (compared to full-length) in vivo is as yet undetermined. In vitro experiments, however, suggest that factor $VIII_{des-797-1562}$ may be more susceptible to proteolytic attack. This makes it tempting to speculate that the B domain may function to protect the functional domains of factor VIII from proteolysis that may occur as the result of low amounts of circulating active protease, perhaps thrombin. This would ensure that factor VIII is activated only at the site of vascular injury where local protease activity would be high. While the physiological function of this region is as yet undetermined, the results of this study show that this region in factor VIII is not required either for the expression of factor VIII coagulant activity or for its interaction with vWF in vitro.

Acknowledgments

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Registry No. Thrombin, 9002-04-4; blood coagulation factor VIII, 9001-27-8.

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